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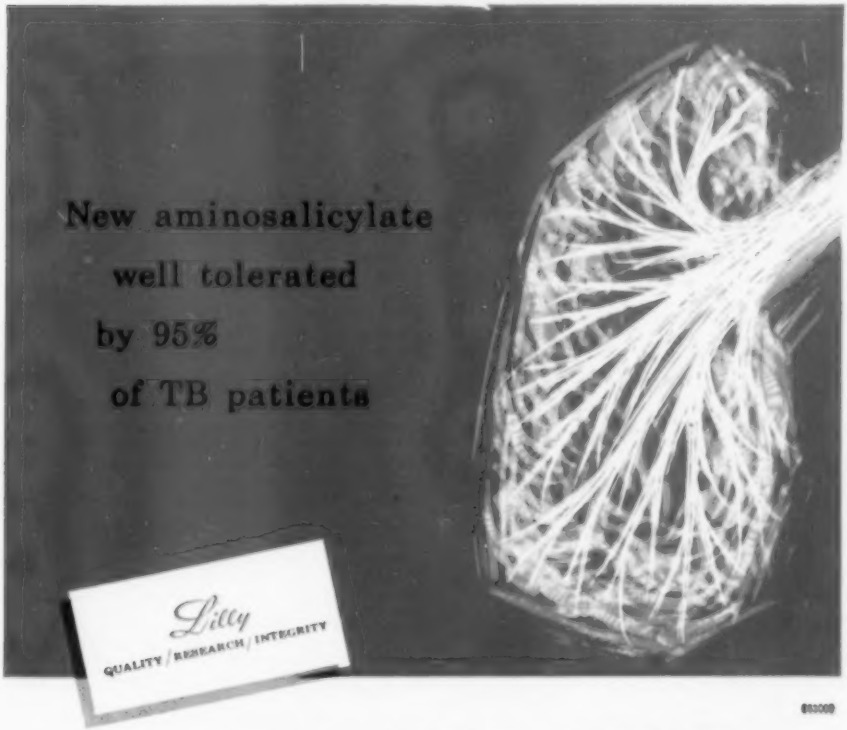

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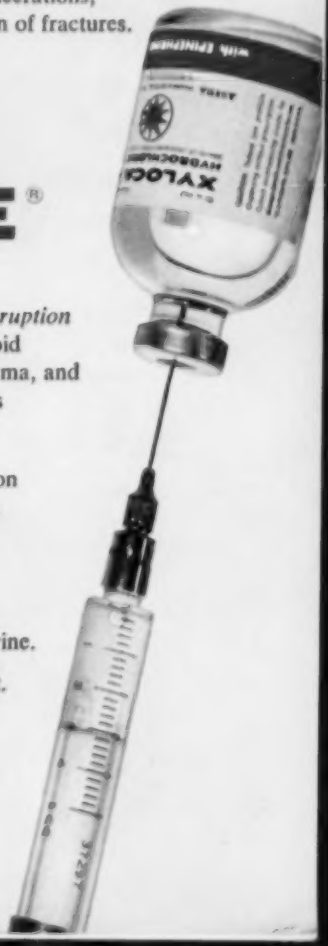

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*Southworth, J. L., and Dabbs, C. H.: Xylocaine: a superior agent for conduction anesthesia, Anesth. & Analg. *32*:159 (May-June) 1953.

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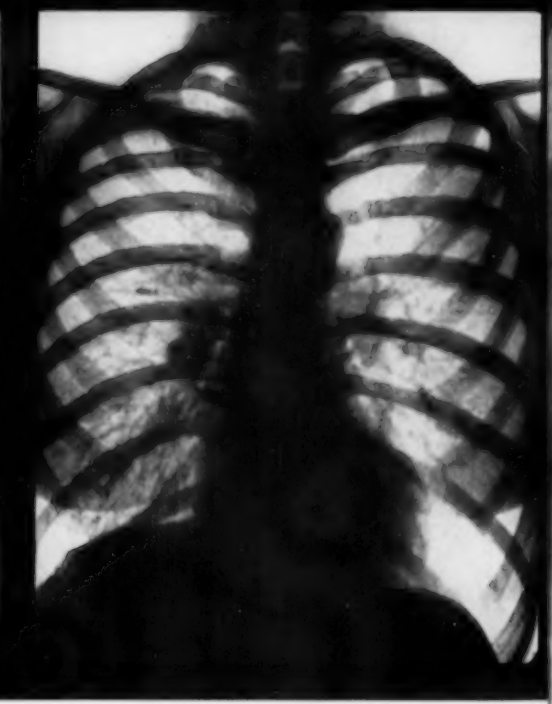

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1. Burrascano, J. J.: Sea View Hosp. Bull. *15*:149 (July) 1955.

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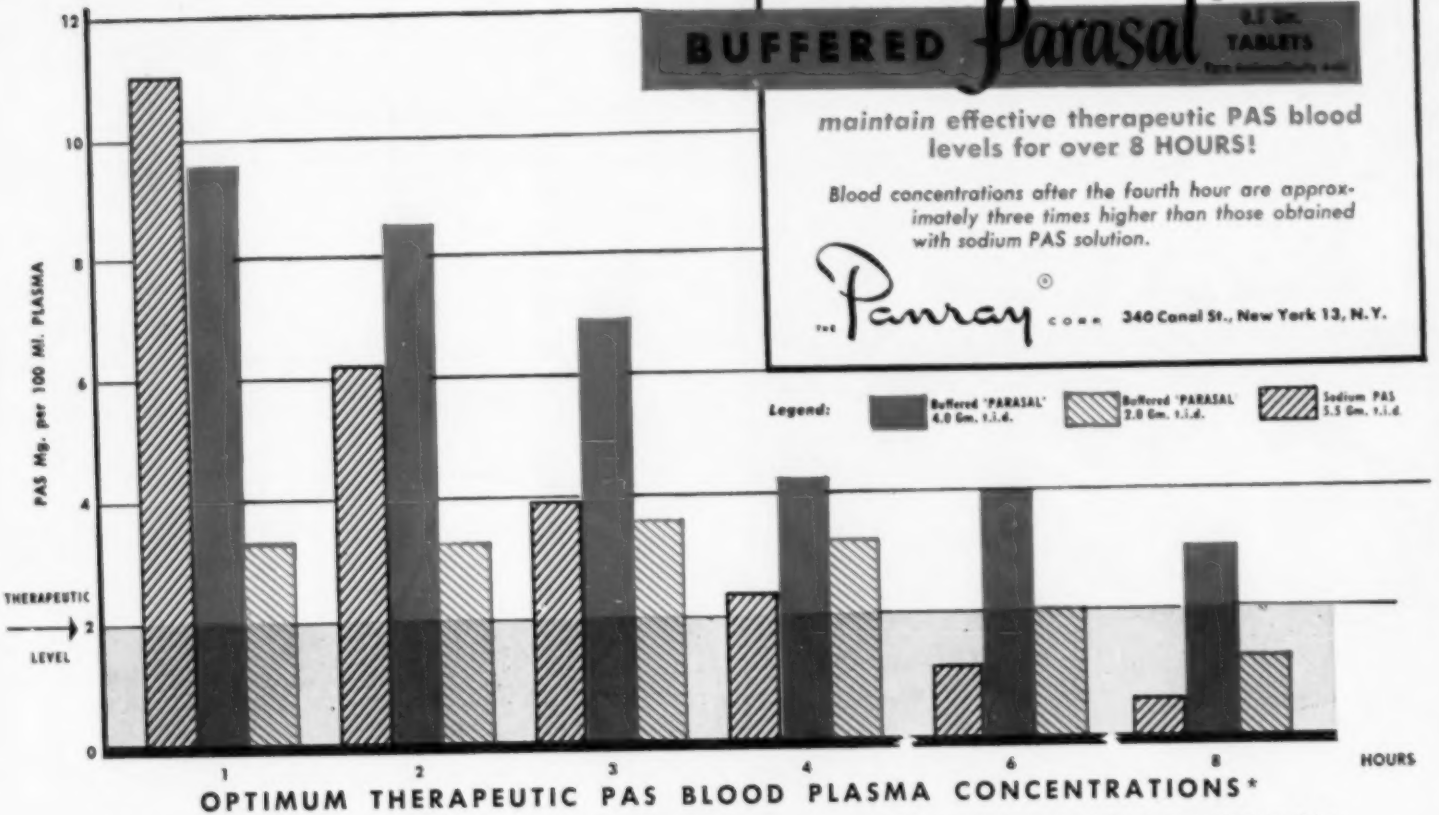

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Ref.: Deeb, E. N. & Vitagliano, G.R.: A. Rev. Tuber. & Pul. Dis.: 72,543-7 (Oct. '55)

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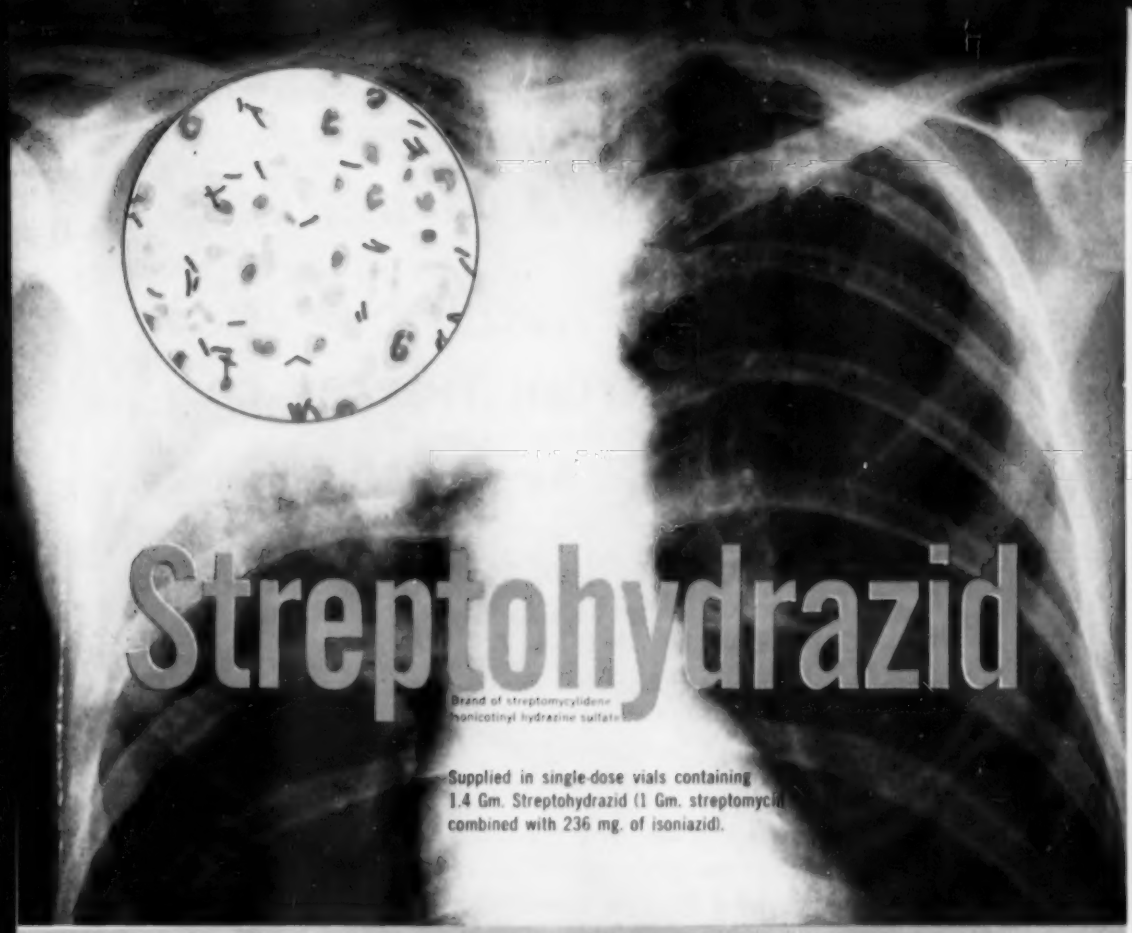

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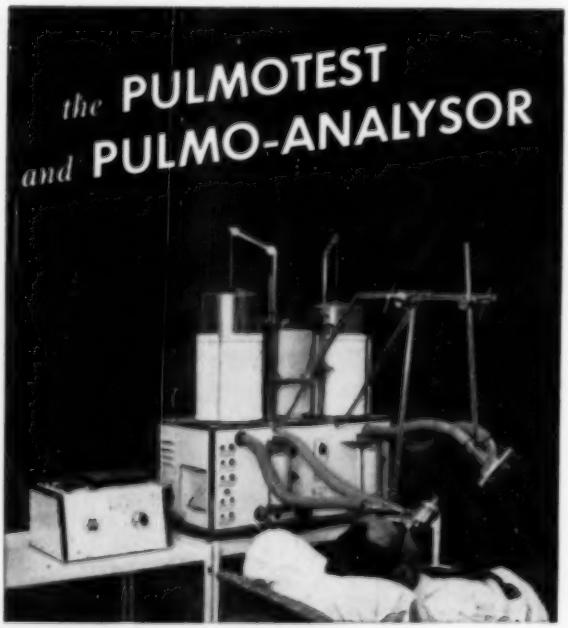

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[1]Dr. Samuel Phillips: *Calcium Benzoyl PAS*, Paper presented at 15th VA-Army-Navy Conference on Chemotherapy of Tuberculosis, St. Louis, Missouri, February 6-9, 1956.

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DIFFERENTIAL CHARACTERISTICS IN VITRO AND IN VIVO OF SEVERAL SUBSTRAINS OF BCG[1]

I. Multiplication and Survival *in Vitro*

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INTRODUCTION

The amount of bacillary material in the dose of BCG vaccine injected intradermally into human beings for the purpose of immunization is of the order of 0.1 mg. or less. This amount is far too small to produce an antigenic stimulus sufficient to elicit immunity to virulent infection. Effective immunization depends, therefore, upon the multiplication of the BCG organisms in the tissues of the vaccinated subject. In fact, many independent lines of evidence indicate that BCG does multiply extensively in man as well as in experimental animals, giving rise to a true infection, although self limited in extent and duration.

It has long been recognized that the extent to which BCG multiplies *in vivo* varies from one batch of vaccine to another and is conditioned, in part, by characteristics inherent in the culture used for the preparation of the bacillary suspension (9, 15, 19, 22, 23, 25, 30, 31, 33, 42, 45). Indeed, published studies from this laboratory have brought out striking differences in morphologic characteristics and in behavior *in vivo* among three substrains of BCG obtained from American institutions (9, 15, 27, 33, 33*a*, 42).

The present series of papers constitutes an extension of these earlier studies and brings together observations made during the past eight years on eleven substrains of BCG obtained from vaccine-producing laboratories in different parts of the world. The results leave no doubt that the various cultures maintained under the label BCG, all derived from the strain originally isolated by Calmette and Guérin, now differ markedly in morphologic and cultural characteristics, in ability to survive and multiply *in vivo* and, consequently, in effectiveness as immunizing agents.

MATERIALS AND METHODS

Media: The culture media used in the present studies have been described in previous publications. The following types of media were used: Tween® albumin liquid medium, albumin or oleic acid–albumin agar, and norite agar (13, 20).

[1] From the laboratories of The Rockefeller Institute for Medical Research, New York, New York.

Cultures: The various BCG substrains will be designated as *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, and *K*. They were obtained from the following sources:

A—Dr. J. Aronson, Henry Phipps Institute, Philadelphia. (Culture received in the form of an ampoule of vaccine.) In previous publications from the writers' laboratory this culture is referred to as Phipps strain or BCG-*P* (27, 33, 42).

B—Dr. Sol Roy Rosenthal, Tice Laboratory, Chicago. (Culture received in the form of an ampoule of vaccine.) This substrain was obtained by Dr. Rosenthal in 1934 from the Pasteur Institute, Paris, and designated by him *BL*. It has been referred to in previous publications from the writers' laboratory as Tice strain or BCG-*T* (27, 33, 42).

C—Dr. J. Luis Gomez Pimienta, Mexico City. (Culture Lot No. 57, received on egg slant.)

D—Dr. A. de Assis, Brazil. (Moreau strain—No. 576, received on potato slant.)

E—Dr. Sol Roy Rosenthal, Tice Laboratory, Chicago. (Culture *BLP*[2] received in the form of desiccated vaccine, Lot No. 949.)

F—Dr. J. Ungar, Glaxo Laboratories, England. (Culture received in the form of desiccated vaccine, Batch No. 48.)

G—Dr. Konrad Birkhaug, Albany, New York. (Culture received in the form of an ampoule of vaccine, No. A124.)

H—Dr. K. Tolderlund, State Serum Institute, Copenhagen. (Received in the form of an ampoule of vaccine, No. 968.)

I—Dr. F. Van Deinse, the Pasteur Institute, Paris. (Culture received in the form of an ampoule of vaccine.)

J—Dr. Gosta Vahlne, Bakteriologisha Laboratoriet, Göteborg. (Culture received as Lot F145.)

K—Central State Scientific Control Institute, L. A. Tarasevich, Moscow, Sivtsev-Vrazhek No. 41. Generation No. 170-N- dried *in vacuo*, 22 IV 1955, received from the Pasteur Institute, Paris, 1924.

The following cultures of tubercle bacilli (other than BCG) were also used in the present study:

RIRv (human strain, attenuated)

H37Rv (human strain, virulent)

H37Ra (human strain, avirulent)

These three cultures were obtained through the courtesy of Dr. William Steenken, Jr., from the Standard Culture Depot maintained by the National Tuberculosis Association at the Trudeau Laboratory, Saranac, New York.

MV (Vallée) (bovine strain, virulent)—originally obtained from the Pasteur Institute, Paris, and reisolated in the writers' laboratory from the spleens of infected mice.

The history and the comparative characteristics of these strains have been described in references (27), (33), (39), and (40).

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[2] The *E* substrain, designated *BLP* by Dr. Sol Roy Rosenthal, was received from him only recently, on February 17, 1955, with the request that it be used to replace his original BCG substrain *BL* (*B* in the present series). It is important to note that many of the experiments reported in the present study were carried out with a culture (*BL*) (designated in this paper as *B* substrain) which is no longer used by Dr. Rosenthal for the preparation of BCG vaccine.

The following details of the history of the *BLP* substrain were obtained from a personal conversation with Dr. Rosenthal on August 8, 1955. The substrain *BLP* is a mixture of three parts of the BCG-*BL* substrain and one part of substrain *P*. Both these substrains were obtained by Dr. Rosenthal from the Pasteur Institute but at different times (*BL* in 1934; *P* in 1951). A mixture of the two substrains was made in Dr. Rosenthal's laboratory in the proportions described above. This mixture was frozen and dried, and is being used for the production of the BCG vaccine. A further description of th *BLP* culture can be found in reference (36).

Animals: Albino mice (so-called Rockefeller Swiss strain) were obtained from the colony maintained at the Rockefeller Institute.

Mice of the C57 black and of the dba, line 2, strains were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine.

The mice were approximately four weeks of age when received and were maintained on a diet of bread and milk, or of pellets (Ralston Purina laboratory chow) and water ad libitum unless otherwise specified under individual experiments. Infection tests were carried out by injecting 0.2 ml. of the proper dilution of culture in one of the lateral caudal veins.

Guinea pigs were obtained from the colony of albino animals maintained at the Rockefeller Institute. They received ad libitum a diet of oats, hay, and cabbage.

The techniques used for homogenization of infected organs by means of a Teflon[3] grinder[3] and for enumeration of visible bacilli in the homogenate, have been previously described (33).

OBSERVATIONS

When the various substrains of BCG are cultivated on conventional culture media *in vitro*—either as surface or as submerged growths—their cell division time during the logarithmic phase at 37°C. is approximately fifteen to twenty-five hours, depending upon the nature of the medium. The final yield of bacterial protoplasm recovered per unit volume of culture in standard media is also essentially independent of the origin of the BCG used as inoculum. On the basis of these cultural similarities, it appears at first sight that the various substrains multiply equally well *in vitro* and, therefore, possess identical metabolic endowments. Other types of tests, however, reveal striking differences in their minimal growth requirements and in their ability to survive under certain environmental conditions *in vitro*. Some of these differences will be illustrated in the following experiments.

Differences in Growth Requirements of BCG Substrains

As the standard egg yolk media contain a large variety of complex organic substances, many of which certainly act as nutritional factors, they do not lend themselves to the comparative study of growth requirements of strains. The use of large inocula (as universally employed for the routine transfer of cultures) also interferes with this type of study because the cellular constituents of the inoculum under these conditions are likely to introduce into the new medium metabolic products which may act as growth factors. For these reasons, it has seemed advisable in the present comparative study of BCG substrains to observe the growth of small inocula in culture media of simple composition. This has been facilitated by the recent discovery that the addition of activated charcoal (norite for example) to semisynthetic culture media of the proper composition will allow the growth of small inocula of mammalian tubercle bacilli (20).

Cultures of various strains of mammalian mycobacteria were grown in liquid Tween medium free of albumin. After approximately seven days' incubation at 37°C., drops of 1:1,000 dilutions of these cultures were deposited on the surface of experimental agar media

[3] The homogenizing units used in the present study consist of an especially adapted chuck, Teflon pestles, and uniformly fitted Pyrex tubes. They are now produced commercially by Tri-R Instruments, 24-15 44th Road, Long Island City 1, New York.

TABLE 1

DIFFERENCES IN GROWTH REQUIREMENTS OF ATTENUATED TUBERCLE BACILLI*

Additives to Basal Norite Agar Medium		Effect on Colonial Growth of Indicated Strains		
		BCG-*A*	BCG-*B*	RIRv
	per cent			
Na oleate	0.005	Enhancement	Inhibition	Enhancement
Cholesterol	0.100	None	Enhancement	None
Arginine	0.200	None	Enhancement	None
Casein hydrolysate	0.300	None	Inhibition	Enhancement
Bacto soytone	0.300	Enhancement	Inhibition	Enhancement
Bacto yeast extract	0.300	None	None	Enhancement

* For experimental details see reference (20).

which were also free of albumin, but contained 0.1 per cent norite (for supplementary experimental details, see reference (20)). The influence of certain nutritional factors added to the basal agar on the bacterial growths obtained is presented schematically in table 1.

As seen in table 1, the various mammalian mycobacteria, and the substrains of BCG in particular, exhibited different requirements for growth on agar media free of egg yolk or serum albumin but containing norite.

On the basis of the results summarized in table 1 and more completely described elsewhere (20), it has proved possible to design a norite agar medium which allows abundant growth of all the strains of mammalian mycobacteria so far tested. More recent observations have revealed, however, that strains which readily produced colonies on norite agar when young cultures were used as inoculum failed to grow on this medium when the inoculum consisted of cells which were older or which had been subjected to certain injurious treatments (like exposure to strong alkali or acid, for example). It has been found, moreover, that the various substrains of BCG differed in the rapidity with which they lost the ability to form colonies on norite agar medium in the course of aging. This is illustrated in the following experiment.

The three BCG substrains *A*, *B*, and *K* were grown in liquid Tween-albumin medium. After different periods of incubation at 37°C., graded dilutions of the cultures were made in liquid norite medium (0.01 per cent norite) and were plated simultaneously on norite agar, on albumin agar, and on Löwenstein's egg media. The numbers of colonies which developed on these three types of solid media are recorded in table 2.

As seen in table 2, the three BCG substrains tested grew readily on norite agar when the inoculum was derived from very young cultures (five days old). Under these conditions, the numbers of colonies which developed on norite agar were only slightly smaller than those obtained on albumin agar or on Löwenstein egg yolk medium. It soon became apparent, however, that aging of culture *B* resulted in a marked decrease in the numbers of colonies produced on all media; cultures *A* and *K* appeared far less susceptible than culture *B* from this point of view.

Even more striking was the difference of behavior observed on norite agar

TABLE 2

EFFECT OF AGING OF CULTURE ON COMPARATIVE ABILITY OF THREE BCG SUBSTRAINS TO MULTIPLY ON DIFFERENT TYPES OF SOLID MEDIA

BCG Substrain	Age of Inoculum	Numbers of Colonies* Obtained on		
		Norite Agar	Albumin Agar	Löwenstein
	days			
A	5	2.4×10^7	6.8×10^7	6.2×10^7
A	10	5.2×10^3	7.8×10^7	7.2×10^7
A	15	8.8×10^4	9.1×10^6	8.2×10^6
B	5	7.9×10^6	1.7×10^7	3.8×10^7
B	10	0	5.4×10^6	2.8×10^6
B	15	0	4.1×10^3	3.3×10^4
K	5	7.9×10^7	1.2×10^8	8.8×10^7
K	10	3.3×10^7	7.1×10^7	6.2×10^7
K	15	1.5×10^5	4.2×10^6	5.8×10^6

* Calculated per ml. of culture

On this medium, culture *B* completely failed to grow after it had been allowed to age for ten days or more, even though it was still capable at that time of growing on albumin agar and on egg yolk media. Substrain *A* exhibited a somewhat analogous behavior in this regard, older cultures of it growing much less abundantly on norite agar than on the albumin or egg yolk media.[4] In contrast, culture *K* continued to grow almost as readily on norite agar as on albumin agar or on Löwenstein egg yolk even after it had been allowed to age for fifteen days in the liquid Tween medium.

It may be seen in table 2 that culture *B* gave an initial viable population (as measured by colonial enumeration on albumin agar) somewhat smaller than that of culture *A*. It may be seen also that the *B* cells died more rapidly than the *A* cells in the original culture medium. Similar differences in behavior between these two strains have been observed under other conditions, as appears from the results of the following experiments.

Substrains *A* and *B* were grown in Tween-albumin medium. While the cultures were in the logarithmic phase of growth, 0.5 ml. samples of each were inoculated separately into 5 ml. of the same medium; this medium was distributed into test tubes closed with aluminum caps. The new cultures were incubated at 37°C. without agitation. The numbers of viable units after different periods of incubation were determined by plating appropriate dilutions on albumin agar (table 3).

[4] The fact that culture *A*, which had lost the ability to grow on norite agar after ten days' aging, recovered this ability on further incubation has been observed repeatedly and can hardly be due to experimental inconsistencies. It appears worth while, therefore, to suggest the following hypothesis to account for this apparent anomaly. There is no doubt that many of the bacteria in culture *A* underwent autolysis during aging, as shown by the appearance of many nonacid fast forms. It is likely, furthermore, that a secondary bacterial growth took place in the aging culture as a result of the stimulatory effect of the release of cellular products during autolysis. It can be postulated that the new cells thus produced would behave as a young inoculum and, therefore, accounts for the ability of the fifteen-day-old culture of substrain *A* to grow on norite agar.

TABLE 3

EFFECT OF TIME OF INCUBATION ON DENSITY AND VIABILITY OF CULTURES OF TWO BCG SUBSTRAINS

Incubation Time	Optical Density		Numbers of Colonies* Recovered from	
	BCG-*A*	BCG-*B*	BCG-*A*	BCG-*B*
days				
5	0.180	0.168	6.8×10^7	1.7×10^7
10	0.218	0.208	7.8×10^7	5.4×10^6
15	Not done	Not done	9.1×10^6	4.1×10^5
21	Not done	Not done	5.4×10^5	2.2×10^3

* Calculated per ml. of culture.

In table 3 it may be noted that, when large inocula of cultures of BCG-*A* and BCG-*B* in the logarithmic phase of growth were introduced into liquid Tween-albumin medium, they multiplied rapidly and reached maximal optical density in approximately the same period of time. The similarity in rate of growth is reflected in the fact that optical density and numbers of colonies yielded on albumin agar were of the same order of magnitude for the two cultures after five days' incubation. In reality, the results of measurement of optical density as well as of viable population suggest that BCG-*A* grew a little more rapidly than did BCG-*B*. This finding is in agreement with the fact, illustrated in table 1, that *B* seems somewhat more exacting than *A* in its growth requirements.

Although the differences seen in table 3 are not large, they are beyond the range of experimental error and have been confirmed in many other experiments. Real as they are, however, these differences are small in comparison with the striking contrast in the numbers of viable units shown to be present in the two cultures after longer periods of incubation. Whereas large numbers of colonies could still be recovered from culture *A* after ten, fifteen, and twenty-one days of incubation, the viability of BCG-*B* fell precipitously during the same period. Indeed, it has been the constant experience in this laboratory that stock cultures of BCG-*B* in Tween-albumin medium yielded much smaller numbers of viable cells than would be expected from their optical density, unless the culture is very young. In general, the majority of the older cells of BCG-*B* fail to multiply on albumin agar and on egg media, and seem to be inactive *in vivo*.

Effect of Anaerobiosis and Aeration on Survival of BCG

As the cultures studied in the preceding experiment were nonagitated, the bacterial cells settled in the bottom of the test tubes. It is probable, therefore, that their local environment became essentially anaerobic during the later phases of incubation when the bacterial growth was sufficiently abundant to consume all of the oxygen that reached the bottom of the tube by diffusion. It has been shown elsewhere that tubercle bacilli die rapidly when placed in an anaerobic atmosphere, particularly if the environment contains carbon dioxide and lactic acid (6, 7). It appeared of interest, therefore, to make comparative studies of the viability of several BCG strains under anaerobic conditions.

TABLE 4

SURVIVAL OF TUBERCLE BACILLI* UNDER ANAEROBIC CONDITIONS†

Strain	Number of Colonies Recovered per Ml. of Culture After			
	5 days	10 days	15 days	20 days
MV	2×10^9	9×10^7	8×10^6	1×10^6
H37Rv	6×10^7	2×10^7	4×10^6	8×10^5
RIRv	7×10^7	7×10^7	1×10^6	8×10^4
BCG-*A*	2×10^8	3×10^7	5×10^6	4×10^5
BCG-*B*	1×10^9	7×10^4	2×10^1	0
H37Ra	5×10^6	9×10^4	1×10^1	0

* Six-day-old cultures in Tween-albumin medium were placed in anaerobic jar; viable cells were enumerated at intervals of time by plating appropriate dilutions on albumin agar medium.

† This, and similar experiments, were carried out in collaboration with Dr. David Weiss.

Two substrains of BCG, namely *A* and *B*, were studied in comparison. The virulent bovine strain MV and human strain H37Rv, as well as the attenuated human strain RIRv and the avirulent H37Ra, were also included in the tests. All cultures were grown in liquid Tween-albumin medium. After six days' incubation several samples of cultures were placed in jars rendered anaerobic by a method permitting liberation of carbon dioxide in the atmosphere during the removal of oxygen (20). The jars were placed in an incubator at 37°C. At various intervals of time, some of the jars were opened and the cultures tested by quantitative bacteriologic techniques to determine the numbers of bacteria that yielded colonies on albumin agar. The results are presented in table 4.

The results presented in table 4 confirm the earlier finding, reported elsewhere (6, 7), that anaerobic environments are not favorable for the survival of mammalian mycobacteria. The results bring out, furthermore, that the various strains of mycobacteria differ in their susceptibility to the lethal effect of anaerobiosis. It may be seen in table 4 that H37Ra and BCG died much faster than the virulent bacilli and that RIRv occupied an intermediate position between the two groups. Of particular interest, for the present study, is the fact that a striking difference could be recognized between the two substrains (*A* and *B*) of BCG. In this experiment as in the preceding one, the cells of substrain *B* lost their viability much more rapidly than did those of *A*; indeed, they failed to yield subcultures after a few days' exposure in the anaerobic environment.

The same cultures used in the experiment reported in table 4 were also studied with regard to their ability to survive in deficient media strongly aerated by violent agitation. As may be seen in table 5, marked differences in viability were recognized under these conditions; moreover, the order of relative resistance to extreme aerobiosis of the various cultures was the same as that shown in table 4 for the effect of anaerobiosis.

Effect of Tween and of Oleic Acid on Growth of BCG

In many other experiments it was also found that the various substrains of BCG differed in susceptibility to the growth inhibitory effect of a number of

TABLE 5

THE SURVIVAL OF TUBERCLE BACILLI IN MINERAL SOLUTION UNDER HIGHLY AEROBIC CONDITIONS*

Strain	Number of Colonies† Recovered After		
	Initial	6 days	30 days
MV	2×10^6	1×10^6	2×10^5
H37Rv	3×10^6	1×10^6	3×10^4
RIRv	3×10^6	4×10^4	1×10^6
BCG-*A*	4×10^6	2×10^5	7×10^4
BCG-*B*	4×10^5	2×10^4	1×10^2
H37Ra	3×10^5	6×10^2	3×10^1

* This, and similar experiments, were carried out in collaboration with Dr. David Weiss.

† Washed bacterial cells were resuspended in balanced ionic mineral solution at pH 6.8; suspension was placed in shaking machine at 27°C. Samples were taken for enumeration of bacilli after six and thirty days' shaking.

well-defined chemical substances: penicillin, dyes, lactic acid, fatty acids, detergents, et cetera. It will suffice to present briefly some of the findings with oleic acid and with Tween 80.

Two types of agar media were prepared: one, the norite medium, described in reference (20); the other having the composition described in reference (13). Graded concentrations of oleic acid or of Tween were added to these media, ranging from 0.04 per cent to 0.002 per cent. Bovine albumin (in the form of plasma fraction V) was added to all media in a final concentration of 0.5 per cent. The albumin agar media were inoculated by deposition on their surface of drops of graded dilutions of young cultures of mycobacteria in Tween-albumin media. The presence of growth and the type of colonies were observed after two weeks' incubation at 37°C. In table 6 is presented the minimal concentration of oleic acid, or of Tween, that brought about partial inhibition of growth of the inocula containing approximately 10^6 viable units. The concentration of Tween 80 that was required to cause the mycobacteria to grow on the agar media in the form of smooth colonies is also indicated in this table.

As seen in table 6, the virulent cultures proved more resistant than the attenuated cultures to the inhibitory effect of either oleic acid or Tween 80 in albumin agar media. Among BCG substrains, the relative order of susceptibility was the same for the two substances. Although not directly related to the subject of the present paper, the effect of Tween 80 on colonial morphology is nevertheless worth noting. As reported in an earlier publication, the addition of Tween 80 to albumin agar media causes the colonies of all the mycobacteria tested in this laboratory to grow with a smooth glistening surface. The minimal concentration of Tween 80 that produces this effect, however, varies from strain to strain. The results presented in table 6 confirm and extend earlier observations in revealing that a far higher concentration of Tween was required to render the colonies of virulent strains smooth than was the case with the attenuated or avirulent strains. Marked differences were also observed among BCG substrains in this regard. Moreover, comparison with the findings to be reported

TABLE 6

COMPARATIVE SUSCEPTIBILITY OF VARIOUS STRAINS OF MYCOBACTERIA TO THE PRESENCE OF OLEIC ACID OR TWEEN 80 IN ALBUMIN AGAR MEDIA

Inoculum (Approximately 10^6 Viable Units)	Concentration (in Per Cent Weight of Medium) Required to Produce		
	Partial Inhibition of Growth		Smooth Colonies Tween 80†
	Oleic Acid*	Tween 80†	
MV	>0.040	>0.040	0.032
H37Rv	0.040	0.032	0.016
RIRv	Not done	0.020	0.008
BCG-*A*	0.030	0.020	0.004
BCG-*B*	0.005	0.002	0.002
BCG-*C*	0.020	0.008	0.004
BCG-*D*	0.010	0.002	0.002
BCG-*E*	0.010	0.004	0.004
BCG-*F*	0.010	0.008	0.004
BCG-*H*	0.020		
BCG-*J*	0.010	0.002	0.002
BCG-*K*	0.010	0.002	0.002
H37Ra	Not done	>0.040	0.002

* In norite albumin agar (20).

† In ordinary albumin agar (13).

in the second and third papers of this series suggests that the BCG substrains which grow in a virulent-like serpentine pattern, and which are highly invasive *in vivo*, also require higher concentrations of Tween 80 to produce smooth glistening colonies on albumin agar media.

DISCUSSION

Many of the substrains of BCG listed in this report have been maintained for several years as stock cultures in this laboratory, both on Löwenstein-Jensen egg medium and in liquid Tween-albumin medium. As a result of numerous attempts to determine quantitatively the ability of these cultures to produce colonies on albumin agar, and more recently also on norite agar, it has become obvious that the various substrains exhibit highly characteristic and predictable growth propensities *in vitro* and, therefore, differ in their metabolic endowments. It has not been possible for lack of time to investigate in a systematic manner the comparative behavior of all of the substrains *in vitro*. Suffice it to say that the few quantitative studies carried out in this laboratory have confirmed the conclusions derived from routine observations.

By the use of graded inocula containing small numbers of cells, it has been shown that the cultures of various substrains of BCG differ profoundly in their growth requirements and in their ability to survive under semianaerobic or totally anaerobic conditions. They differ also with regard to the concentration of oleic acid or of Tween that they can tolerate in the culture medium. Other types of metabolic differences have recently been brought out in more limited comparative studies of BCG substrains (7, 9, 26, 41, 44).

It may seem surprising at first that these facts have not been more widely recognized in the past and, indeed, that experienced students of BCG have insisted that the differences observed are merely phenotypic, rather than of a genotypic nature. Three reasons readily account for the frequent lack of awareness of the genetic variability of BCG. First is the fact that most laboratories devoted to the study of these organisms carry only one strain, under the assumption that BCG is a "virus fixe" and that subcultures of it differ only as a result of phenotypic phenomena elicited by cultural conditions. More shall be said concerning this matter in the Discussion of the fourth paper of the present series.

Among other reasons that have interfered with the recognition of differences among substrains, one might mention the narrow range of environmental circumstances and of culture media used for the study of BCG. On the one hand, the universal practice to grow the bacilli on surfaces does not provide any opportunity to observe susceptibility to the deleterious effect of partial or complete deprivation of oxygen. On the other hand, egg yolk or bile-potato media are of such complex organic composition as to provide little or no chance to detect differences in growth requirements. It is true that BCG is grown on fairly simple media for the final large-scale production of the vaccine. But in this operation, as well as in those involved in the routine maintenance of stock cultures, it is the practice to use inocula containing millions or billions of living cells. These enormous bacterial masses all but obliterate the differences that are revealed when inocula containing much smaller numbers of cells are used, as in the present study. In other words, the techniques which have proved practicable for the routine transfer of stock cultures and for the preparation of vaccine are not well suited to bring to light cultural differences among substrains of BCG.

Doubt may be expressed concerning the relevance of the facts discussed in the present paper to the use of BCG as a vaccinating agent against tuberculosis. True enough there is as yet no evidence that growth requirements *in vitro* have any bearing on behavior *in vivo*. There are a number of facts, on the other hand, which suggest that susceptibility to the toxic effects of anaerobiosis can be related to the ability of the cultures to multiply and to persist *in vivo*. As pointed out elsewhere (9, 11, 12), the inflammatory area is essentially anaerobic and is the site of accumulation of lactic acid, as a result of the glycolytic activity of inflammatory cells. In consequence, one can postulate that strains which rapidly lose their viability in anaerobic environments containing lactic acid are not likely to persist long in the tissues after immunity and allergy have become established, since these immunologic phenomena call forth an intense inflammatory response around the bacilli at the site of infection. Indeed, it will be shown in the third paper of this series that the substrains of BCG that are most susceptible to anaerobiosis *in vitro* are also those that disappear most rapidly from the tissues of infected animals.

SUMMARY

The various substrains of BCG differ in their ability to multiply and to survive in laboratory culture media. These differences of behavior *in vitro* have been

investigated in the present study by quantitative bacteriologic determinations which have revealed the following facts:

The number and size of colonies yielded by graded inocula on norite agar differ from substrain to substrain, and are influenced by the presence of amino acids, protein hydrolysates, oleic acid, and cholesterol in the basal medium.

The substrains of BCG in albumin media differ also with regard to the concentration of oleic acid and of Tween®, as well as of many antibacterial agents that they can tolerate.

As a result of aging, the BCG cultures (as well as virulent cultures) progressively lose the ability to produce colonies on norite agar; they remain capable for a much longer period of growing on albumin agar or on egg yolk media. The loss of ability to produce colonies on norite agar occurs much more rapidly with certain BCG substrains than with others.

The BCG strains differ profoundly in the length of time during which they remain viable in liquid Tween-albumin medium.

The BCG strains also exhibit striking differences in their ability to survive in the absence of oxygen, a fact which probably affects their ability to persist *in vivo* at the site of the inflammatory reaction.

Observations are presented suggesting that the effect of Tween on colonial morphology varies from substrain to substrain and is related to invasiveness *in vivo*.

SUMARIO

Características Diferenciales In Vitro e In Vivo de Varias Subcepas del BCG: I. Multiplicación y Sobrevivencia In Vitro

Las varias subcepas del BCG discrepan en su capacidad para multiplicarse y sobrevivir en los medios de cultivo del laboratorio. Esas diferencias de comportamiento *in vitro* han sido investigadas en el estudio actual con determinaciones bacteriológicas cuantitativas que han revelado los siguientes hechos:

El número y el tamaño de las colonias rendidas por inóculos graduados en agar-norita discrepan de subcepa en subcepa y son afectados por la presencia de ácidos amínicos, hidrolisatos de proteína, ácido oleico y colesterina en el medio básico.

Las subcepas del BCG también discrepan con respecto a la concentración de ácido oleico y de Tween®, así como de muchos agentes antibacterianos, que pueden tolerar en medios de albúmina.

A consecuencia del envejecimiento, los cultivos del BCG (e igualmente los virulentos) pierden progresivamente la capacidad para producir colonias en agar-norita; pero permanecen capaces por mucho más tiempo de proliferar en medios de agar-albúmina o de yema de huevo. La pérdida de la capacidad para formar colonias en agar-norita ocurre con rapidez mucho mayor en ciertas subcepas del BCG que en otras.

Los subcultivos del BCG discrepan profundamente en el tiempo que permanecen viables en el medio de Tween-albúmina líquido.

También manifiestan notables diferencias en su capacidad para sobrevivir privados de oxígeno, hecho este que afecta probablemente su capacidad para persistir *in vivo* en el sitio de la reacción inflamatoria.

Preséntanse observaciones que sugieren que el efecto del Tween sobre la morfología colonial varía de subcepa en subcepa y se relaciona con la facultad invasora *in vivo*.

RESUME

Caractères différentiels in vitro et in vivo de diverses souches dérivées du B.C.G.: I. Reproduction et survie in vitro

Les sous-souches dérivées du BCG diffèrent quant à leur aptitude à se reproduire et à demeurer viables dans les milieux de culture *in vitro*. Les différences de comportement *in vitro* ont été étudiées dans le travail actuel par des déterminations bactériologiques quantitatives qui ont révélé les faits suivants:

Le nombre et les dimensions des colonies obtenus par des inocula étalonnés sur milieu gélose-norite diffèrent d'une sous-souche à l'autre et sont influencés par la présence d'acides aminés, d'hydrolysats protéiques, d'acide oléique et de cholestérol dans le milieu de base.

Dans les milieux à l'albumine les sous-souches dérivées du BCG diffèrent également quant à la concentration d'acide oléique et de Tween ainsi que de nombreux agents antibactériens qu'elles peuvent tolérer.

En vieillissant, les cultures de BCG (de même que les cultures virulentes) perdent progressivement leur capacité de produire des colonies sur milieu gélose-norite; elles restent capables plus longtemps de se développer sur les milieux gélose-albumine ou au jaune d'oeuf. L'aptitude à produire des colonies sur gélose-norite se perd beaucoup plus rapidement dans le cas de certaines sous-souches dérivées du BCG que d'autres.

Les sous souches du BCG diffèrent considérablement en ce qui concerne la durée de leur viabilité dans le milieu liquide Tween-albumine.

Elles presentent aussi de grandes differences quant à leur temps de survie en l'absence d'oxygène, fait qui influence probablement leur aptitude à persister *in vivo* au siège de la réaction inflammatoire.

Les auteurs présentent des observations qui permettent de penser que l'effect du Tween sur la morphologie des colonies varie d'une soussouche à l'autre, et est en relation avec le pouvoir des germes de se reproduire *in vivo*.

(The relevant bibliography will be found at the end of the fourth paper of this series.)

DIFFERENTIAL CHARACTERISTICS IN VITRO AND IN VIVO OF SEVERAL SUBSTRAINS OF BCG[1]

II. Morphologic Characteristics *in Vitro* and *in Vivo*

CYNTHIA H. PIERCE AND RENÉ J. DUBOS

(Received for publication August 11, 1956)

INTRODUCTION

It is now well recognized that the mammalian mycobacteria exhibit one of two very distinct morphologic patterns of growth (26–28, 32, 44, 46). As far as is known, all of the virulent strains rapidly form on the surface of liquid media a thin veil which spreads uniformly over the entire surface and climbs high on the sides of glass containers. In contrast, some (although not all) of the avirulent and attenuated mutants have less tendency to spread on the surface; they produce instead discrete islands which coalesce only late in growth. Studies of microscopic morphology, both in liquid and on agar media, have revealed furthermore that the virulent bacilli grow according to an impressively oriented pattern, serpentine in appearance, with the rods arranged in parallel in the direction of the long axis of the bacillary chains. Under the same conditions, the avirulent bacilli grow in clumps in a nonoriented, "helter-skelter" fashion.

These contrasting morphologic patterns of growth express themselves in characteristic colonial morphology on oleic-acid–albumin agar media. Cultures which grow with a serpentine pattern (virulent-like) give rise to spreading colonies with a thin flat wavy margin. In contrast, the other cultures (H37Ra-like) form heaped up nonspreading colonies.

It will be shown in the present paper that the various substrains of BCG differ among themselves with reference to their morphologic characteristics. Some are almost indistinguishable in this respect from the fully virulent strains of mammalian mycobacteria, whereas others exhibit a poorly oriented or nonoriented type of growth similar to that of the avirulent variants. Furthermore, the cultures of most, if not all, BCG substrains appear to be mixtures of several morphologic types in different proportions.

OBSERVATIONS

Morphologic Characteristics of Colonial Growth Yielded by Stock Cultures of BCG Substrains

The extreme morphologic types of colonial growth exhibited by mammalian mycobacteria (virulent and avirulent) are illustrated in figure 1. This figure presents the colonial morphology on oleic-acid–albumin agar of colonies of the virulent human strain H37Rv (*a*), of its avirulent mutant H37Ra (*b*), and of the virulent bovine strain, MV (*c*).

[1] From the Laboratories of The Rockefeller Institute for Medical Research, New York, New York.

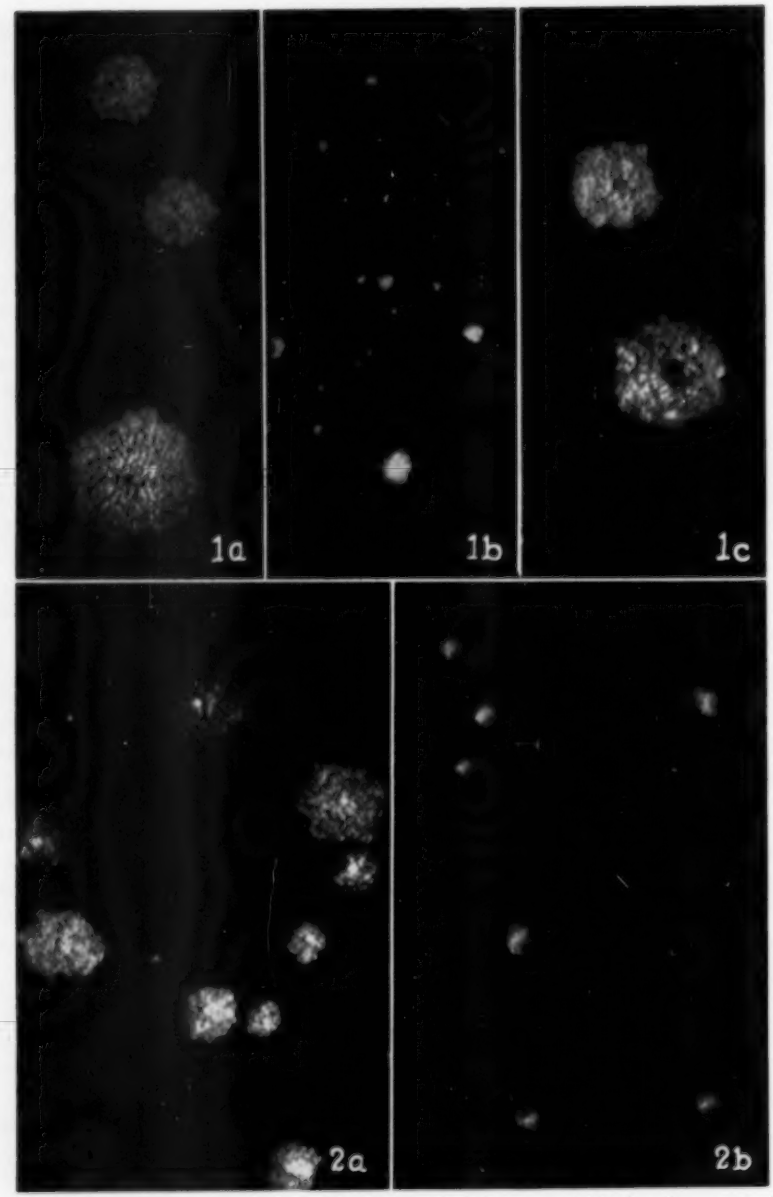

FIG. 1. Colonies, two weeks old, on oleic-acid-albumin agar. (*a*) Virulent human strain, H37Rv. (*b*) Avirulent variant, H37Ra. (*c*) Virulent bovine strain, MV. (Magnification 25—reflected light).

FIG. 2. Colonies, two weeks old, on oleic-acid-albumin agar. (*a*) BCG (substrain *C*). (*b*) BCG (substrain *D*). (Magnification 25—reflected light).

These three strains were grown for ten days in liquid Tween®-albumin medium; drops of dilutions of the growth were then deposited on the surface of the agar. The colonies were photographed after two weeks' incubation at 37°C.

As may be seen in figure 1, both virulent strains (H37Rv and MV) produced large colonies with a serpentine pattern of growth, spreading at the periphery, whereas colonies of the avirulent variant (H37Ra) were smaller in size and had no obvious tendency to spread.

In figure 2 are presented the colonial characteristics of two different substrains of BCG (*C* and *D*) studied under conditions identical with those used for the test illustrated in figure 1. After two weeks' incubation, the colonies of BCG-*C* (*a*) on oleic-acid–albumin agar resembled those of the virulent strains, whereas the colonies of BCG-*D* (*b*) were more similar to those of avirulent strains.

In figure 3 are compared the colonies produced on oleic-acid–albumin agar by cultures in liquid albumin medium of the two BCG substrains, *A* (*a*) and *B* (*b*). In this case, the colonies were observed after four weeks' incubation, this longer period of growth accentuating even more the difference between the two substrains. Clearly, BCG-*A* resembled in morphology the virulent cultures, whereas BCG-*B* was more like an avirulent culture.

As pointed out in an earlier publication, most BCG substrains are heterogeneous to varying degrees, as shown by the fact that their cultures yield on

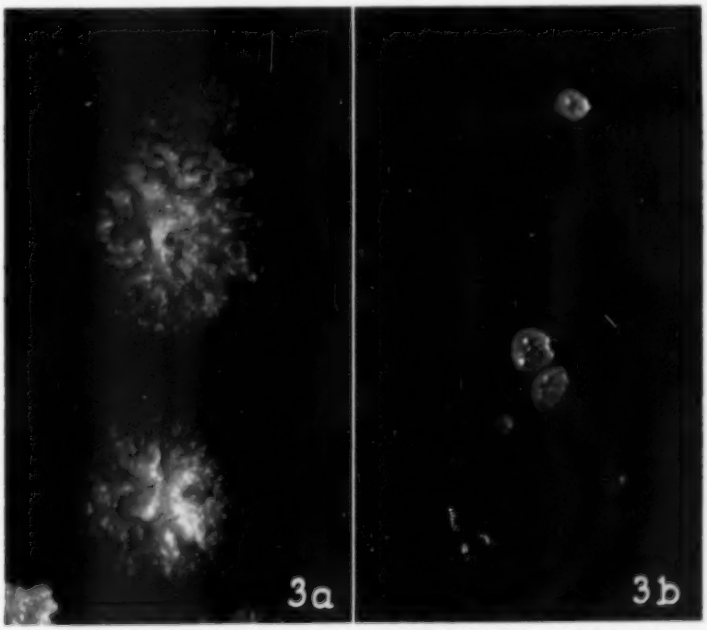

FIG. 3. Colonies, four weeks old, on oleic-acid–albumin agar. (*a*) BCG (substrain *A*). (*b*) BCG (substrain *B*). (Magnification 8.3—reflected light).

albumin agar several morphologic types of colonies (42). Although the relative proportions of the different morphologic types appear to remain fairly constant for each culture under uniform conditions of cultivation, these proportions may vary when the culture is changed from one environment to another.

The morphologic heterogeneity of BCG-*F* is illustrated in figures 4 and 5. In figure 4 the appearance (in transmitted light) of spreading and nonspreading colonies obtained from a culture of BCG-*F* in Tween-albumin liquid medium is shown. The colonies were two weeks old on albumin agar. Although only one spreading colony can be seen among many more nonspreading forms in figure 4*a*, the proportion of the former type has been found to increase after several subcultures in Tween-albumin medium (figure 4*b*).

The appearance on norite agar medium of two-week-old colonies of BCG-*E* and BCG-*F* (in comparison with those of the virulent bovine culture MV) is shown in figure 5. Whereas the colonies of BCG-*E* (figure 5*a*) are hardly distinguishable from those of the virulent culture (figure 5*b*), it may be seen that BCG-*F* (figure 5*c*) yielded a mixed population of spreading and nonspreading forms.

Effect of the Presence of Tween in the Culture Medium on the Mode of Growth of Mycobacteria

It has been shown elsewhere that the colonial mode of growth of mycobacteria can be altered by addition of Tween to albumin agar (13*a*, 27, and the first paper of the present series), this wetting agent giving to the colonies on albumin agar a smooth appearance and nonspreading character. Tween also interferes with the formation of strands of cells in liquid media (26, 44). It has been found also that the concentration of Tween required to produce these effects is greater for the virulent strains of mycobacteria than for their attenuated mutants. There is evidence, furthermore, that differences can be detected from this point of view among the various attenuated mutants (RIRv and substrains of BCG): the more attenuated (less invasive) the culture, the smaller the concentration of Tween required to cause the bacilli to grow in a completely disoriented manner.

As reported in the preceding paper of the present series, these observations have recently been extended to other cultures of mammalian mycobacteria. It has been shown (table 6 of Paper I) that the concentration of Tween required in albumin agar to produce smooth colonies varies from 0.032 per cent for the highly virulent bovine strain MV to 0.002 per cent for the avirulent culture H37Ra and for certain substrains of BCG. Other substrains of BCG require concentrations intermediate between these two extremes. As will be shown in the following paper, there exists a relationship between the concentration of Tween required to render the colonies smooth and glistening and the degree of attenuation of the substrain.

Stability of Morphologic Characteristics of Substrains of BCG

The observations presented so far leave no doubt that the various substrains of BCG studied in this laboratory differ profoundly in morphologic character-

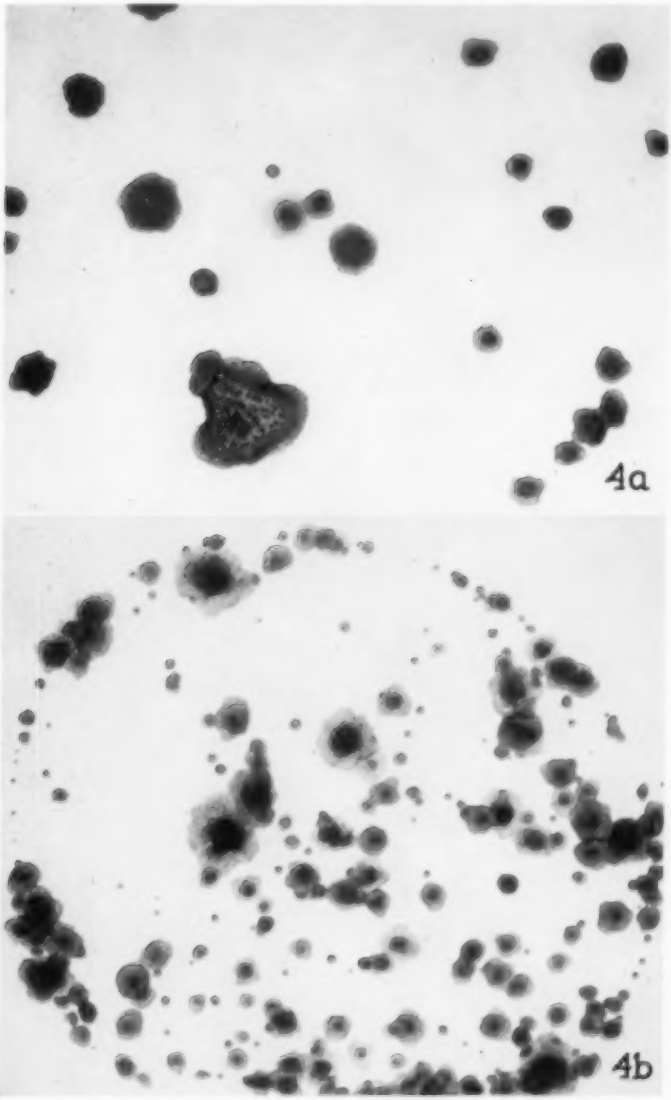

FIG. 4. Colonies, two weeks old, on albumin agar. (*a*) BCG (substrain *F*). (*b*) BCG (substrain *F*) after several subcultures in Tween-albumin medium. (Magnification 8—transmitted light).

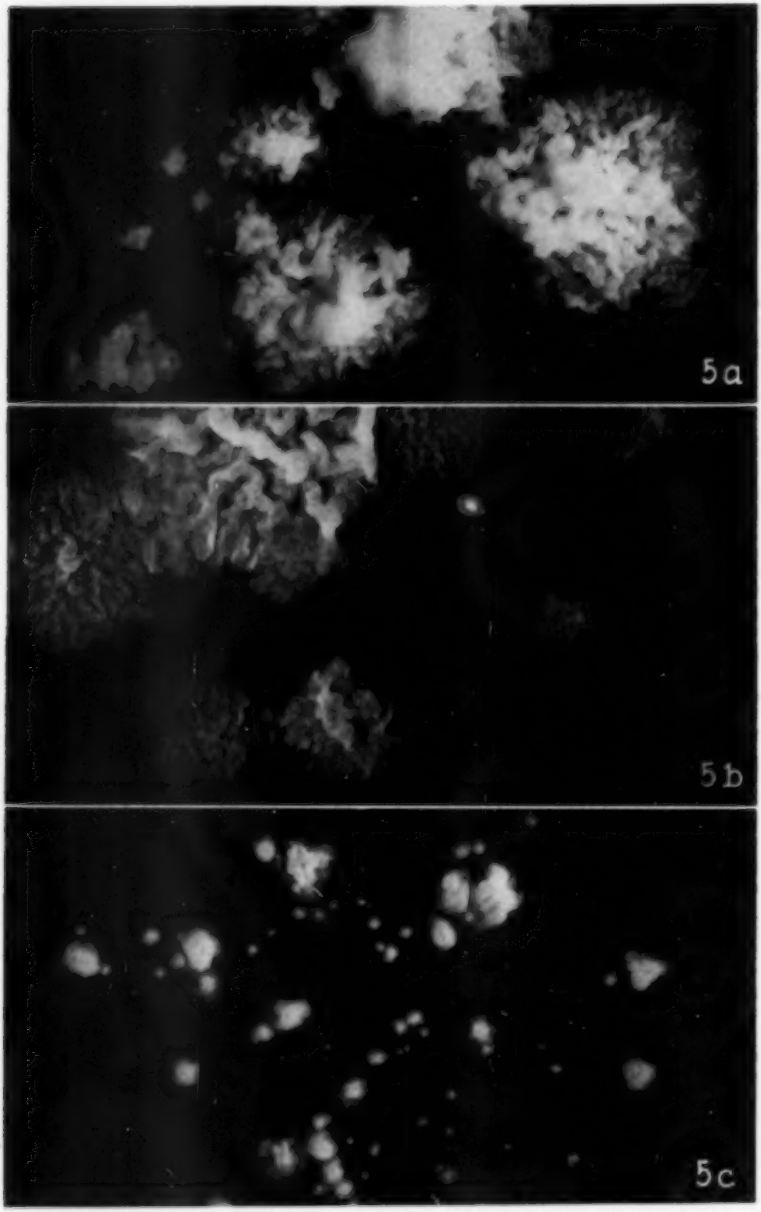

FIG. 5. Colonies, two weeks old, on norite agar. (*a*) BCG (substrain *E*). (*b*) Virulent bovine strain, MV. (*c*) BCG (substrain *F*). (Magnification 8.8—reflected light).

istics and in homogeneity. As has been described, the various substrains yield, on albumin or on oleic-acid–albumin agar, as well as on norite agar, colonies which range in appearance from that typical of the most virulent forms to that exhibited by the avirulent variants (H37Ra-like). Since the observations previously reported on this subject were made with BCG cultures that had been subcultured repeatedly in Tween-albumin medium, it appeared useful to investigate the growth patterns of cultures not subjected to this method of cultivation but, instead, recovered directly from the BCG used by the vaccine manufacturers.

Immediately upon receipt from the vaccine-producing laboratories, the BCG cultures were plated in serial dilutions, either directly on to oleic-acid–albumin agar if received in a liquid medium, or after resuspension in diluent if received in the lyophilized state. The findings under these conditions are illustrated in figure 6.

In figure 6 may be seen the appearance in transmitted light of colonies obtained by plating on albumin agar suspensions in distilled water of lyophilized samples

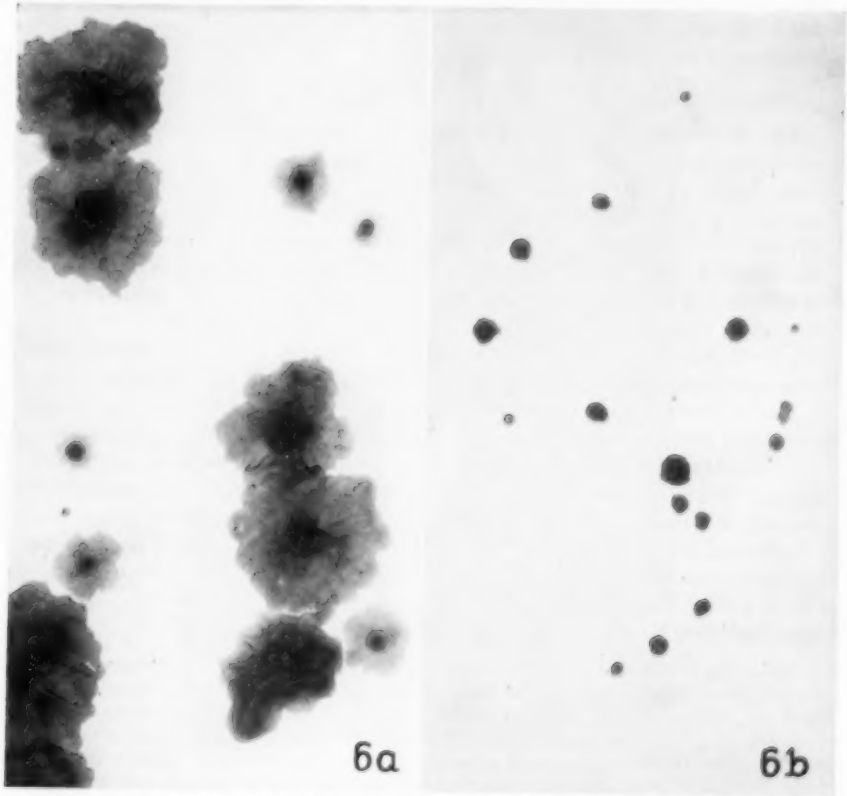

FIG. 6. Colonies obtained from lyophilized vaccine samples, two weeks old, on oleic-acid–albumin agar. (*a*) BCG *E*. (*b*) BCG *F*. (Magnification 8—transmitted light).

of BCG-*E* and BCG-*F* vaccine. After two weeks' incubation, the colonies of BCG-*E* (figure 6*a*) were large with a spreading periphery, whereas those of BCG-*F* (figure 6*b*) were small and nonspreading. Thus, the colonial morphology yielded by the two bacterial suspensions under uniform conditions was profoundly different, even though dilutions and inoculations on agar had been made directly from the vaccine preparations without passage through Tween medium.

In general, it may be said that, upon receipt from the vaccine-producing laboratories, the various substrains could be classified into two groups on the basis of colonial morphology. Cultures of substrains *A*, *C*, *G*, *E*, *H*, *I*, *J*, and *K* were similar in general appearance and their colonial morphology resembled that seen in figures 2*a* and 3*a*. The colonial morphology of substrains *B*, *D*, and *F* was of the type represented in figures 2*b* and 3*b*. As will be shown in the following paper, this differentiation was confirmed by comparative studies of multiplication in mice. It must be mentioned here that the substrains *A* and *B* have remained stable throughout many subcultures over a period of years in this laboratory, whereas the substrains *D* and *F* have exhibited some variation with respect to colonial morphology.

Alterations in Morphologic Characteristics Brought About by in Vivo Environment

The differences that have been observed among the cultures of BCG certainly correspond to inherent characteristics of the various substrains. Indeed, it has been shown in a previous publication from this laboratory (42) that, when amounts ranging from 0.03 to 0.1 ml. of culture of substrains *A*, *B*, or *G* are injected into guinea pigs or mice, the growth recovered on albumin agar from the organs of these animals sacrificed one to twelve weeks after injection consists of mixtures of colonies. The mixtures contain the same relative distribution of spreading, intermediate, and nonspreading types as that occurring in the culture originally injected. Under these conditions, therefore, the *in vivo* environment does not seem to exert a selective effect on the different colonial types.

More recent studies, however, have provided evidence that the *in vivo* environment can influence colonial morphology under certain special circumstances. This effect was observed in experiments in which 0.2 ml. of BCG culture was injected intravenously into mice which were then subjected to intermittent periods of fasting, and were sacrificed four weeks after infection. Under these conditions, it was found in several animals that the proportion of spreading to nonspreading colonies recovered from the lungs greatly exceeded that occurring in the culture injected. This effect either failed to take place in the spleen or was overshadowed by circumstances which were not clearly understood.

Albino mice of the so-called Rockefeller Swiss strain, three weeks old, received intravenously 0.2 ml. of an eight-day-old culture of BCG *D* in liquid Tween-albumin medium. The mice were kept on a pellet diet, and glucose was added to the drinking water in a final concentration of 5 per cent. They were fasted for forty-eight hours during the first week and again during the second week after infection by withholding the pellets. They were sacrificed four weeks after infection, and homogenates of their spleens and lungs were plated in serial dilutions on albumin agar.

The results of the experiment are shown in figures 7 *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, and *i*.

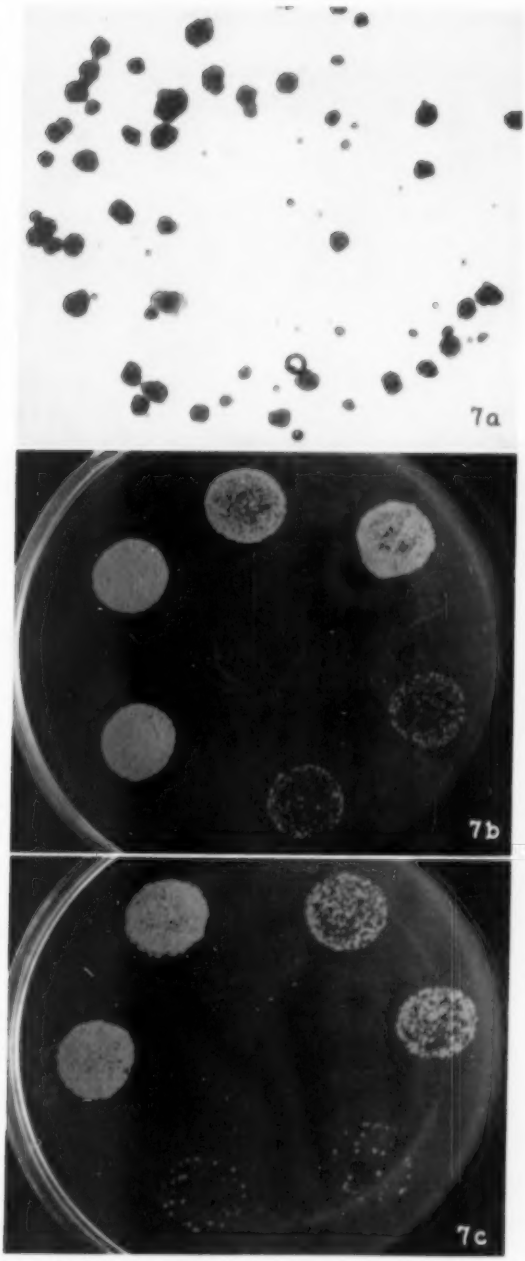

FIG. 7. Colonies of BCG-*D*, two weeks old, on albumin agar. (*a*) BCG-*D* (Magnification 8—transmitted light). (*b*) BCG-*D* obtained from serial tenfold dilutions (duplicates) of infected spleen homogenate. (*c*) BCG-*D*, obtained from serial tenfold dilutions (duplicates) of infected lung homogenate. (Magnification 1.3—reflected light).

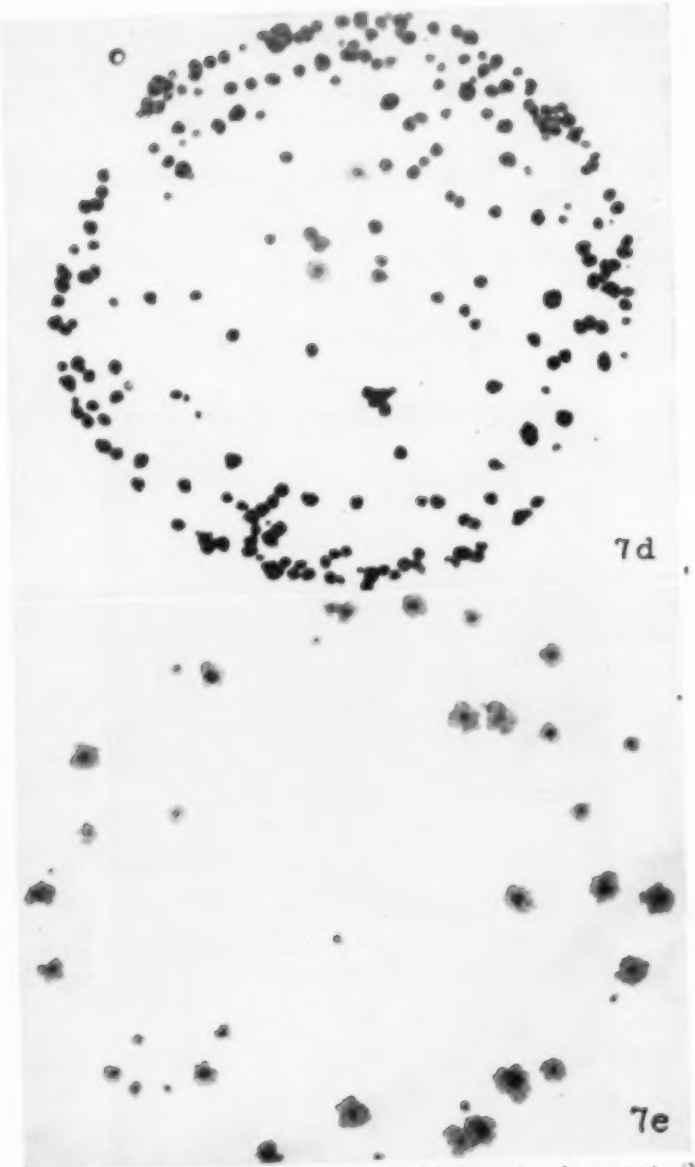

FIG. 7. (*d*) BCG-*D* from spleen homogenate. (*e*) BCG-*D* from lung homogenate. (Magnification 8.3—transmitted light).

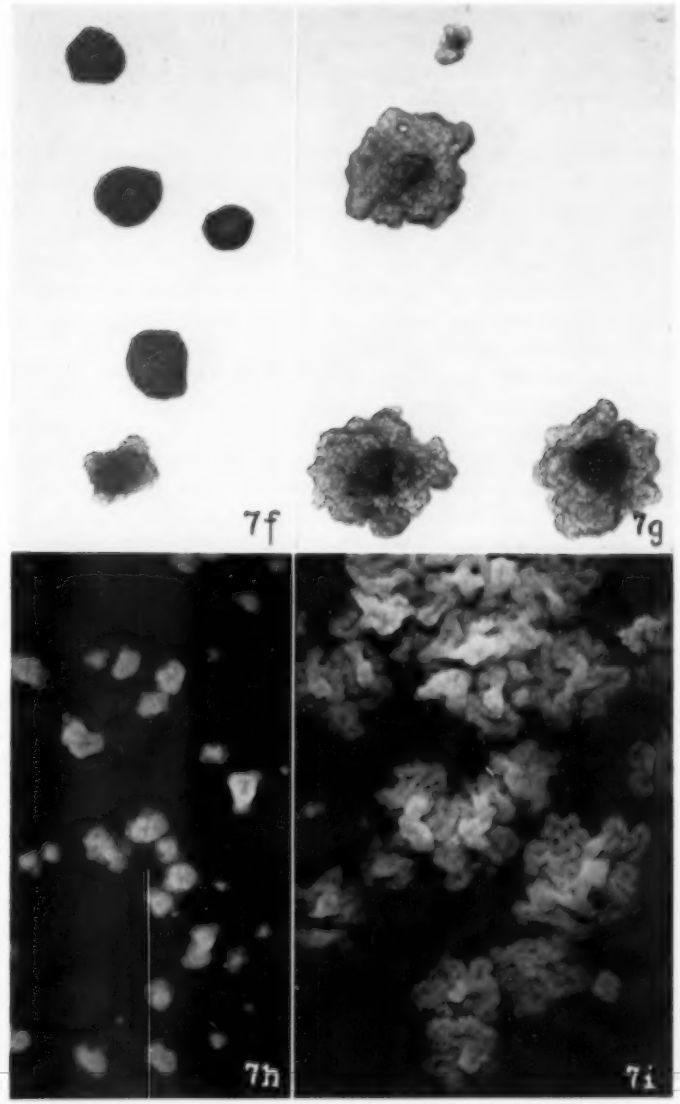

FIG. 7. (*f*) BCG-*D* from spleen homogenate. (*g*) BCG-*D* from lung homogenate. (Magnification 33—transmitted light). (*h*) Colonies obtained from infected spleen homogenate. (*i*) Colonies obtained from infected lung homogenate. Photographs (*h*) and (*i*) represent colonies of BCG-*D*, two weeks old, on norite agar. (Magnification 8.8—reflected light).

On albumin agar the culture used for infection appeared somewhat heterogeneous with reference to colonial morphology. Although it yielded predominantly nonspreading colonies after two weeks' incubation at 37°C., a few spreading forms also appeared (figure 7*a*). In figures 7*b* and 7*c* are shown the appearance of colonies obtained on albumin agar by duplicate platings of serial tenfold dilutions of spleen and lung homogenates from a mouse sacrificed four weeks after infection. In figures 7*d* and 7*e* may be seen the colonies from the same homogenates at higher magnifications. It may be seen that in the final dilution all of the colonies recovered from the lungs had a spreading periphery, whereas the majority of those obtained from the spleen homogenate were nonspreading. In figures 7*f* and 7*g*, which are still higher magnifications of individual colonies obtained from spleen and lung homogenates, the morphologic differences between them are demonstrated more sharply.

In a later experiment carried out according to the same general plan, the homogenates were plated on norite agar as well as albumin agar. The type of growth obtained on norite agar is shown in figures 7*h* and *i*. As in the preceding experiment, they reveal a striking difference in the type of colonies recovered from the spleen, 7*h*, and from the lung, 7*i*, the latter organ yielding a growth with marked spreading at the periphery.

Similar results have been obtained from other experiments in which 0.2 ml. of culture of BCG-*F* was used as the infective inoculum.

The experiments *in vivo* just described indicate that some factor of the environment in mouse lungs favors the growth of BCG in the spreading form. The following experiments were designed to test whether the effect of this "lung factor" could also manifest itself *in vitro*.

The spleens and lungs of normal mice were ground individually in 2 ml. of a 0.1 per cent solution of albumin in physiologic saline. To 2 ml. of tissue homogenate there was added 0.1 ml. of an undiluted ten-day-old culture of BCG-*B* in liquid Tween-albumin medium. Graded dilutions of this mixture were plated on oleic-acid–albumin agar medium immediately after mixing, and again after three and six days' incubation at 37°C.

It will be recalled that, under normal conditions, only nonspreading colonies are obtained when cultures of BCG-*B* are plated on albumin or oleic-acid–albumin agar. In contrast, it may be seen in figure 8*a* that, when a suspension of this culture was plated immediately after mixing with lung homogenate, it yielded a number of large spreading colonies which constituted approximately 4 per cent of the total growth. These colonies were not obtained from the bacterial suspension in the spleen homogenate, nor, of course, from the original culture. There was no evidence that prolonged incubation of the culture with the tissue homogenates changed the results appreciably.

Further analysis of the phenomenon has revealed that the large spreading colonies can be evoked just as readily by using the clear supernatant obtained by centrifugation of this material instead of the total lung homogenate. The phenomenon is illustrated in figure 8*b*. As was to be expected, the supernatant of the spleen homogenate was inactive in this regard.

A few studies have been made of the comparative behavior of the two types

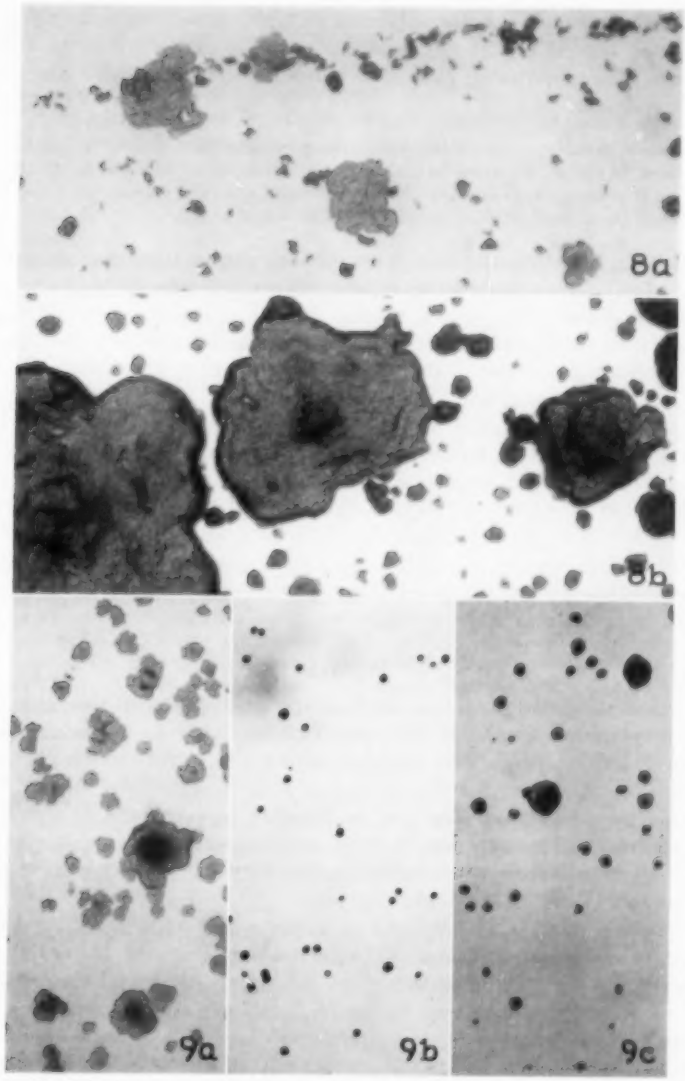

FIG. 8. (*a*) Colonies, two weeks old, on oleic acid–albumin agar; Tween albumin culture of BCG *B* treated with lung homogenate (Magnification 13—transmitted light). (*b*) Tween-albumin culture of BCG *B* treated with supernatant from lung homogenate. (Magnification 55—transmitted light).

FIG. 9. Colonial morphology of BCG *B* emulsified in (*a*) lung extract, (*b*) 0.1 per cent albumin, (*c*) spleen extract. (Magnification 13—transmitted light).

of colonies recovered from the substrain BCG-*B*, namely, those exhibiting the nonoriented type of growth characteristic of the stock culture of the substrain and those with a spreading pattern obtained as a result of prior contact with lung homogenate.

Colonies of these two types were emulsified separately with a Teflon® grinder either in lung extract, in spleen extract, or in 0.1 per cent albumin. Serial dilutions in the respective diluents of the suspensions were then plated on albumin agar. The appearance of the colonial growth after two weeks' incubation at 37°C. is illustrated in figure 9.

Here again, it was found that large colonies with a spreading pattern grew from the two bacterial suspensions in lung extract, 9*a*, whereas both suspensions in albumin diluent, 9*b*, yielded smaller, nonspreading colonies. The growth obtained from the suspensions in spleen extract, 9*c*, exhibited a colonial morphology intermediate between those resulting from contact of the bacterial suspensions with lung extracts and with albumin solution.

Although the information available does not warrant a discussion of the mechanisms of these phenomena or of their significance, two facts which have relevance to them may be mentioned in passing. It has been observed, on the one hand, that the ability of lung homogenate to evoke the appearance of spreading colonies is lost when this material is diluted a few fold. On the other hand, all experiments designed to test the effect of lung homogenate on the avirulent culture H37Ra have so far failed to bring about the appearance of spreading colonies from this avirulent mutant.

SUMMARY

A comparative study has been made of the type of colonial morphology produced by eleven substrains of BCG on albumin or oleic-acid–albumin agar.

Some of the substrains were found to exhibit a serpentine pattern of growth, resulting in colonies with a flat spreading margin, which was similar to that produced by the virulent strains of mammalian mycobacteria.

In contrast, other substrains grew in a poorly oriented manner, producing heaped-up colonies without spreading margin, very similar to those of the avirulent mutants (H37Ra-like).

The colonies of all BCG substrains on albumin agar could be rendered smooth and glistening by adding Tween® 80 to the medium. However, the concentration of Tween required to produce this effect differed greatly from substrain to substrain; it appeared to be higher for the BCG cultures still endowed with a high degree of invasiveness.

Most substrains were heterogeneous to varying degrees, yielding mixtures of the different morphologic types in fairly constant proportions.

Each of the substrains retained its morphologic characteristics unaltered for several years under uniform methods of laboratory cultivation. However, morphologic changes could be induced by the proper manipulations *in vitro* and *in vivo*.

Evidence was obtained that there exists in the lungs of mice a factor as yet

unidentified which favors the spreading type of colonial growth. Increase in multiplication, or in survival, of the bacilli with the spreading type of growth has been achieved (*1*) *in vivo* by injecting large doses of BCG culture into mice subjected to nutritional disturbances and recovering the culture from lung tissue; (*2*) *in vitro* by exposing the bacilli to homogenate of mouse lung or the supernatant of the homogenate for a short time before cultivating them on albumin agar.

SUMARIO

Características Diferenciales In Vitro e In Vivo de Varias Subcepas del BCG: II. Características Morfológicas In Vitro e In Vivo

Este estudio comparado versó sobre el tipo de la morfología colonial producida por once subcepas del BCG en agar-albúmina o ácido oleico albúmina.

Algunas de las subcepas mostraron un patrón serpentino de desarrollo, dando por resultado colonias de borde plano de expansión, que era semejante al producido por las cepas virulentas de micobacterias de mamífero.

En contraposición, otras subcepas se desarrollaron en forma desorientada, produciendo colonias sin borde de expansión, muy semejante a los de las mutantes avirulentas (como H37Ra).

Agregando Tween 80® al medio, podían convertirse en lisas y brillantes a las colonias de todas las subcepas del BCG. Sin embargo, la concentración de Tween requerida para lograr este efecto discrepaba considerablemente de subcepa en subcepa, pareciendo ser mayor para los cultivos del BCG dotados todavía de mucha facultad invasora.

La mayoría de las subcepas era heterogénea en mayor o menor grado, rindiendo mezclas de los diversos tipos morfológicos en proporciones bastante constantes.

Cada una de las epas retuvo sus características morfológicas inalteradas durante varios años con métodos uniformes de cultivo en laboratorio. No obstante, cabía inducir alteraciones morfológicas con las debidas manipulaciones *in vitro* e *in vivo*.

Obtuviéronse pruebas de que existe en los pulmones de los ratones un factor todavía por identificar que favorece la forma propagadora de colonias. Con esta forma propagadora de colonias, se ha obtenido aumento de la multiplicación, o de la sobrevivencia de los bacilos: (*a*) *in vivo* inyectando grandes dosis de cultivo de BCG en ratones sometidos a trastornos nutritivos y recuperando el cultivo del tejido pulmonar; (*b*) *in vitro* exponiendo los bacilos a un homogeneato de pulmón murino o a la porción sobrenadante del homogeneato durante un breve período de tiempo antes de cultivarlos en agar-albúmina.

RESUME

Caractères différentiels in vitro et in vivo de diverses souches dérivées du B C G.: II. Caractères morphologiques in vitro et in vivo

Cet article présente une étude comparative du type morphologique des colonies produites sur gélose-albumine ou acide oléique-albumine par onze sous-souches dérivées du BCG.

Certaines de ces sous-souches ont présente un type de croissance sinueuse formant des colonies s'étalant en surface, ressemblant à celles produites par les souches virulentes bovines et humaines.

Au contraire, d'autres sous-souches se sont développées selon une orientation mal définie en produisant des colonies mamelonnées à bords non étalés, ressemblant aux mutants avirulents (comme le H37Ra).

Les colonies de toutes les sous-souches dérivées du BCG cultivées sur gélose-albumine ont pu être rendues lisses et brillantes par l'addition de Tween 80 au milieu. La concentration de Tween nécessaire pour obtenir cet effet a varié d'une sous-souche à l'autre; étant

plus élevée pour les cultures de BCG douées d'un haut degré de pouvoir envahissant *in vivo*.

La plupart des sous-souches se sont montrées hétérogènes à différents degrés, donnant des mélanges de types morphologiques variés en proportions relativement constantes.

Chacune des sous-souches a conservé sans changement appréciable ses caractéristiques morphologiques pendant plusieurs années, dans les conditions uniformes de culture au laboratoire. Néanmoins, des changements morphologiques ont pu être effectués par certains traitements *in vitro* et *in vivo*.

On a mis en évidence, dans le poumon de la souris, un facteur encore non identifié qui favorise le développement des colonies en voile. Cet effet a été obtenu (a) *in vivo*, par l'injection de doses élevées de cultures de BCG à des souris soumises à des troubles nutritionels et (b) *in vitro* en exposant les bacilles à l'homogénat de poumon de souris ou au liquide surnageant de cet homogénat avant de les cultiver sur gélose-albumine.

Acknowledgment

All photographs were taken by Mr. Julian Carlile of The Rockefeller Institute for Medical Research.

(The relevant bibliography will be found at the end of the fourth paper of this series.)

DIFFERENTIAL CHARACTERISTICS IN VITRO AND IN VIVO OF SEVERAL SUBSTRAINS OF BCG[1]

III. Multiplication and Survival *in Vivo*

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INTRODUCTION

A few studies have been published suggesting that the various substrains of BCG do not all behave alike *in vivo* (5, 15, 19, 23, 25, 31–33, 42, 45). Foremost among them was the demonstration by K. A. Jensen that the severity and persistence of the lesions produced by injecting standardized doses of BCG bacilli into the skin of guinea pigs differed from one culture to another (22, 23). This finding has been confirmed and extended in an earlier report from this laboratory (42). Quantitative bacteriologic studies have furthermore revealed that the various substrains of BCG differ also in their ability to multiply and to survive in the organs of mice (32, 33).

The experiments to be described in the present paper supplement the information available concerning the behavior of a number of BCG substrains *in vivo*. They have revealed furthermore that some of these substrains are capable of causing progressive disease and death in animals placed under conditions of physiologic stress.

OBSERVATIONS

Lesions Produced in the Skin of Guinea Pigs by Intradermal Injection of BCG

The variability among individual animals naturally makes it difficult to compare quantitatively the severity of the lesions that the various substrains of BCG can produce in the skin of guinea pigs. In an attempt to overcome this difficulty, a technique involving the following two operations was used in the present study:

> (*a*) Graded dilutions of standardized cultures of several substrains were injected in comparable areas of the skin in the same animal; (*b*) the culture dilutions were tested simultaneously in multiple animals, using different permutations of the different substrains.

In practice, it was found convenient to use four culture dilutions of each of two BCG substrains per guinea pig, one of the cultures being injected in the right flank and the other in the left.

Five BCG substrains were used in the comparative tests reported in tables 1 and 2, namely *A*, *B*, *G*, *H*, and *I*. The cultures were grown for eight days in liquid Tween albumin

[1] From the laboratories of The Rockefeller Institute for Medical Research, New York, New York.

TABLE 1

LESIONS PRODUCED IN GUINEA PIG SKIN BY DIFFERENT DILUTIONS OF THREE BCG SUBSTRAINS

BCG Substrain	Culture Dilution	Diameter of Lesions* at Indicated Times			Activity Ratio†
		1 week	2 weeks	3½ weeks	
BCG-*A* vs. BCG-*B*					
A	1:10	10.0-1.0	11.3-1.5	9.2-2.9	A/B = 30
A	1:30	9.7-0.7	10.2-2.5	8.5-1.7	
A	1:100	7.2-0	8.2-2.0	7.5-1.6	
A	1:300	6.0-0	8.5-1.5	8.2-1.6	
B	1:10	6.5-0.2	7.2-0.7	5.7-1.5	
B	1:30	5.2-0.2	6.2-0.1	4.5-0.1	
B	1:100	3.0-0	5.2-0	2.2-0	
B	1:300	2.7-0	2.7-0	0.5-0	
BCG-*A* vs. BCG-*G*					
A	1:10	10.5-0.7	10.7-3.1	9.0-3.3	A/G = 10
A	1:30	7.0-0.5	8.7-2.0	7.7-2.2	
A	1:100	6.0-0	7.5-1.5	7.0-1.7	
A	1:300	4.7-0	7.2-1.5	7.0-2.1	
G	1:10	6.7-0.2	8.2-1.5	6.7-1.0	
G	1:30	5.2-0	7.5-0.7	5.5-0.7	
G	1:100	3.2-0	4.5-0.2	4.0-0.2	
G	1:300	1.0-0	3.7-0	3.0-0	
BCG-*B* vs. BCG-*G*					
B	1:10	7.0-0	8.5-1.2	10.7-2.1	G/B = 3
B	1:30	6.0-0	7.5-0	6.2-1.1	
B	1:100	4.5-0	6.5-0	3.5-0.2	
B	1:300	0-0	3.7-0	3.0-0	
G	1:10	7.0-0	7.2-1.1	5.7-1.2	
G	1:30	5.7-0	6.0-0.1	4.2-0.5	
G	1:100	2.7-0	4.7-0	2.0-0	
G	1:300	0-0	2.2-0	0.5-0	

* The figures indicate the average diameters of the total inflammatory areas and of the central necrotic areas per group of 4 guinea pigs.

† The activity ratio was estimated from the dilutions of each culture (in each comparative test) that gave the most similar lesions.

medium and were then adjusted to approximately the same optical density with sterile medium. The numbers of viable particles in each culture at the time of injection were determined by plating on albumin agar.

Four culture dilutions of each substrain were injected, 1:10, 1:30, 1:100, 1:300; these dilutions were prepared in 0.1 per cent bovine albumin and were injected intradermally in a volume of 0.1 ml.

The guinea pigs were of the albino Rockefeller Institute strain and weighed 400 to 500

TABLE 2

SUMMARY RESULTS OF SEVERAL TESTS COMPARING ACTIVITY OF BCG CULTURES IN SKIN OF GUINEA PIGS

Experiment Number	BCG Cultures Compared	Optical Density	Colonial Count	Activity Ratio*
1	B_1†	0.229	1,500	1
1	B_2†	0.229	1,685	1
2	A_1†	0.155	Not done	50
2	A_2†	0.167	Not done	50
2	B	0.155	Not done	1
2	G	0.167	Not done	5
3	A	0.202	3,150	30
3	B	0.202	2,184	1
3	G	0.202	2,088	3
4	A	0.218	1,550	10
4	B	0.208	1,263	1
4	G	0.208	1,813	3
5	A	0.180	875	10
5	B	0.180	825	1
5	H	0.180	600	4
5	I	0.180	1,100	10

* Estimated in comparison with activity of culture *B* (taken as unit) in each individual test.

† B_1, B_2, A_1, A_2 were two different cultures of BCG-*B* and BCG-*A*.

gm. They were shaved on both flanks and four separate dilutions of one culture were injected into the skin of each flank, the injections being spaced one inch apart. Similar dilutions of the two cultures to be compared were injected symmetrically on the right and left flanks. Four animals were used for each comparative test on two strains.

The skin lesions were observed at weekly intervals. The diameters of the total inflamed area and of the central necrotic area were recorded (in mm.) and arithmetic averages for each group of 4 animals were calculated. The results of one experiment are presented in tables 1 and 2. Figure 1 shows the appearance of the lesions eleven, twenty-five, and thirty-five days after injection.

The results presented in tables 1 and 2 confirm and extend the earlier findings with BCG-*A* and BCG-*B* (42). When the same numbers of viable cells were injected intradermally into guinea pigs, the former substrain (*A*) produced skin lesions much more severe and longer lasting than those produced by the latter (*B*). Cultures of the substrains *G*, *H*, and *I* were intermediate between *A* and *B* in this regard. In terms of the dilutions of each culture giving skin lesions of the same order of severity, substrains *A*, *G*, *H*, and *I* appeared to be more active than BCG-*B*. Comparative titrations by this technique carried out at irregular intervals of time over a period of years in this laboratory have always yielded results permitting arrangement of the various substrains of BCG in the same order of relative activity.

Multiplication of BCG in the Organs of Mice

It has been shown elsewhere that the *A* and *B* substrains of BCG can readily be differentiated by injecting known amounts of cultures into albino mice and

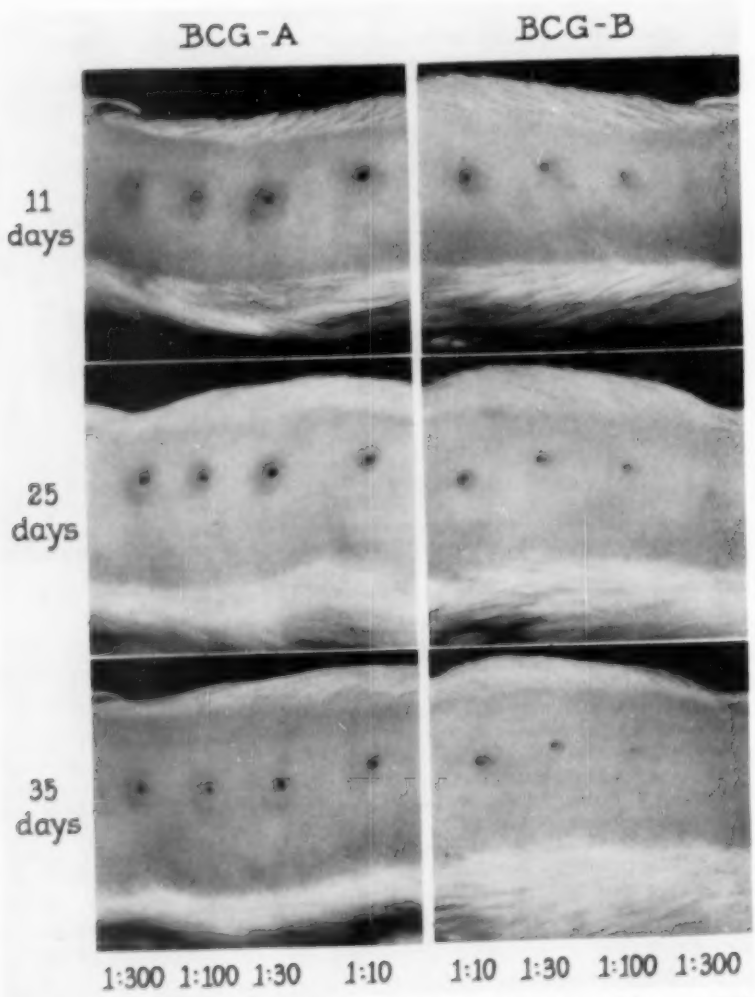

FIG. 1. Evolution of skin lesions in time following injection of graded doses of BCG-*A* or BCG-*B*. Increasing doses of BCG-*A* (1:300, 1:100, 1:30, 1:10) from left to right. Increasing doses of BCG-*B* (1:10, 1:30, 1:100, 1:300) from right to left; 0.1 ml. of culture dilution injected in each case. Photographs taken at eleven, twenty-five, and thirty-five days after injection.

determining quantitatively the number of organisms in the various organs at stated intervals of time after injection (33). The results already published show clearly that BCG-*A* multiplies more abundantly and persists longer in mice than does BCG-*B*. Similar comparative studies have now been carried out with these and other substrains of BCG, as well as with the attenuated human strain, RIRv.

TABLE 3

COMPARATIVE PERSISTENCE OF FOUR BCG SUBSTRAINS IN MICE

BCG Strain Injected*		Numbers† of Colonies Recovered at Indicated Times After Infection		
		6 weeks	8 weeks	12 weeks
A	Spleen	603	400	199
A	Lungs	906	313	18
B	Spleen	45	61	1
B	Lungs	<1	<1	<1
C	Spleen	255	61	176
C	Lungs	103	62	7
D	Spleen	160	47	20
D	Lungs	<1	<1	<1

* Approximately 5,000 viable units of each culture injected intravenously.

† To be multiplied by 100 for whole organ. In this table, as well as in tables 4, 5, 7, and 8, each figure represents the arithmetic average of the numbers of colonies obtained from the organs of 4 individual mice.

In table 3, for example, the results are presented of an experiment designed to test the comparative persistence *in vivo* of four substrains of BCG (*A*, *B*, *C*, *D*), subcultured in Tween-albumin medium. The infective dose in each case was approximately 5,000 viable units. In confirmation of other results, it may be seen that great differences existed among substrains in the rate at which the bacilli disappeared from the organs. BCG-*A* and BCG-*C* obviously persisted much longer than did BCG-*B* and BCG-*D*.

In table 4 are illustrated in greater detail the differences observed *in vivo* between the two BCG substrains *C* and *D*. This comparative study is of special interest because the bacilli used for injection in this case were from cultures which had gone through only one subculture in Tween-albumin medium after receipt from the vaccine-producing laboratory.

In one part of the experiment mice were injected intravenously with 0.2×10^{-3} ml. of seven-day-old cultures in liquid Tween-albumin medium. Groups of 4 animals were sacrificed two days, one week, two weeks, three weeks, four weeks, six weeks, eight weeks, or twelve weeks after infection. In the other part, mice were infected with 0.2 ml. of undiluted culture and were sacrificed twelve weeks after infection. The numbers of colonies recovered from the different organs are presented in table 4.

Examination of the results presented in table 4 show that, immediately after injection, the numbers of bacilli recovered from the spleen and from the lungs were slightly greater in mice which had received BCG-*D* than in those injected with BCG-*C*. Yet, twelve weeks later, the infection caused by BCG-*C* was more severe (as judged from the bacterial population in all organs) than that caused by BCG-*D*, thus confirming the results reported in table 3.

A number of comparative experiments have been carried out to study the behavior in mice of vaccine preparations as received from the producing laboratories, without subcultures in Tween-albumin medium. The range of differences that can be observed between vaccines studied under these conditions is illus-

TABLE 4

EFFECT OF BCG SUBSTRAIN AND OF DOSE OF VACCINE INJECTED ON FATE OF BCG BACILLI IN ORGANS OF MICE

Vaccination		Numbers* of Colonies Recovered at Indicated Times After Injection							
BCG Substrain	Ml.		2 days	1 week	3 weeks	4 weeks	6 weeks	8 weeks	12 weeks
C	0.2×10^{-3}	Spleen	55	108	685	218	76	24	190
C	0.2×10^{-4}	Lungs	<1	3	12	241	42	8	23
D	0.2×10^{-3}	Spleen	210	435	1,018	573	160	48	29
D	0.2×10^{-4}	Lungs	3	4	14	17	0	0	0
C	0.2	Spleen							292
C	0.2	Lungs							702
C	0.2	Liver							537
C	0.2	Kidneys							738
C	0.2	Heart							45
C	0.2	Brain							8
D	0.2	Spleen							62
D	0.2	Lungs							129
D	0.2	Liver							337
D	0.2	Kidneys							6
D	0.2	Heart							20
D	0.2	Brain							2

* To be multiplied by 100 for whole organ.

TABLE 5

FATE IN MICE OF BCG FROM TWO LYOPHILIZED PREPARATIONS

Vaccination		Numbers of Colonies Recovered at Indicated Time After Vaccination				
Strain*	Viable Units Injected		1 week	2 weeks	3 weeks	4 weeks
X	9×10^7	Spleen	3,600	8,450	1,135	1,245
X	9×10^7	Lungs	363	7,050	5,075	9,725
X	9×10^6	Spleen	5	42	143	175
X	9×10^6	Lungs	0	4	14	14
Y	2×10^6	Spleen	33	31	143	1,243
Y	2×10^6	Lungs	0	<1	<1	0

* As the two lyophilized samples compared in this experiment had been obtained from producers using different techniques for the preparation and preservation of the vaccine, it is believed that the results do not provide adequate information concerning the inherent characteristics of the BCG substrains involved. For this reason, it seems wiser not to indicate here the origin of the materials tested.

trated in table 5, which presents the results obtained with lyophilized samples prepared from two different substrains of BCG.

It is necessary to emphasize at this point that the interpretation of comparative tests carried out on preparations of lyophilized vaccines is rendered difficult by the fact that individual samples, even of the same origin, often differ in the numbers of viable organisms that they contain. Furthermore, differences in the

TABLE 6

NUMBER OF VIABLE ORGANISMS IN MICE FED AN INADEQUATE DIET AND INFECTED WITH LARGE INOCULA OF BCG SUBSTRAINS A, B, C, D, OR OF THE ATTENUATED HUMAN STRAIN, RIRv

Strain Injected (0.2 ml. intravenously)	Numbers* of Colonies Recovered from Individual Animals 8 weeks After Injection							
	Spleen				Lungs			
BCG-*A*	53.00	71.00	155.0	—	320.0	115.0	150.0	650
BCG-*B*	0.02	0.03	0.3	5.1	0.2	0.3	0.5	—
BCG-*C*	5.60	31.00	33.0	41.0	5.2	7.0	5.4	19
BCG-*D*	2.80	36.00	45.0	—	9.5	4.3	5.2	—
RIRv	TNC†	TNC	TNC	TNC	TNC	TNC	TNC	TNC

* To be multiplied by 10,000 for whole organ.

† TNC—too numerous to count at final dilution plated (1:100 for spleen; 1:1,000 for lungs).

techniques employed for the production and preservation of the vaccine certainly affect the physiologic state of the surviving organisms and consequently their behavior *in vivo*. For these reasons, it would not be justified to conclude from an examination of table 5 that its results necessarily reflect differences in the genetic, inherent characteristics of the substrains used for the preparation of the lyophilized samples *X* and *Y*.

Behavior of BCG in Experimental Animals Subjected to Nutritional and Metabolic Disturbances

In all experiments reported so far, the studies of the comparative behavior *in vivo* of BCG substrains were carried out with mice maintained under favorable laboratory conditions and fed conventional diets. As will be presently shown, the range of differences that could be detected among substrains of BCG could be widened in certain cases by using animals which were fed diets which have been shown elsewhere to increase susceptibility to bacterial infections (8, 9, 37).

Cultures of RIRv and of substrains *A*, *B*, *C*, and *D* of BCG were grown for seven days in liquid Tween-albumin medium. They were found to contain at the time of use approximately the same numbers of viable cells. Amounts of 0.2 ml. of these cultures were injected intravenously into three-week-old albino mice.

The infected animals were fed a low protein, high carbohydrate, citrate-containing diet of the following composition: white flour, 450 gm.; dry skim milk, 40 gm.; Cerelose®, 400 gm.; sodium citrate, 100 gm.; salt mixture, 10 gm.[‡] These ingredients were resuspended in an equal weight of 7.5 per cent gelatin and the mixture was allowed to gel in the refrigerator. The diet in the form of gelatin cakes and water as drinking fluid were given ad libitum throughout the period of experimentation.

The animals were sacrificed eight weeks after infection and the numbers of mycobacteria in their spleens and lungs were determined by quantitative bacteriologic techniques (table 6).

‡ KCL—42.5 gm., Na_2CO_3—42.5 gm., $MgCO_3$—14.3 gm., $CaCO_3$—25.0 gm., Ferric ammonium citrate—5.5 gm.

As had been found previously with mice which were fed a normal diet, many more organisms were recovered from the organs of animals infected with the attenuated human strain RIRv than from those infected with BCG (table 6). Of the four BCG substrains tested, *A* yielded the largest, and *B* the smallest, numbers of colonies, whereas substrains *C* and *D* were intermediate between these two extremes. It is also clear that the relative order of activity *in vivo* for the five cultures tested was the same whether comparison was based on the bacterial population in the spleen or in the lungs.

Similar differences among substrains have been obtained under other experimental conditions. The following experiment, for example, brings out the marked contrasts in the fate *in vivo* of RIRv and BCG-*B* when these organisms are injected into mice placed on a variety of nutritional regimens.

Cultures of BCG *B* and of the attentuated human strain RIRv were grown for ten days in Tween-albumin medium. Amounts of 0.2 ml. of these cultures were injected intravenously into albino mice three weeks old. Some of the animals were fed ad libitum the low protein-carbohydrate-citrate diet described above. Others were fed pellets ad libitum. A third group received also pellets ad libitum except that the animals were subjected to two periods of two consecutive days of fasting one week and two weeks after infection, during which they were completely deprived of solid food. The animals of the first and second groups received water ad libitum as drinking fluid. The animals of the fasted group received ad libitum a solution of 5 per cent glucose instead of water throughout the period of experimentation.

All animals (4 per group) were sacrificed eight weeks after infection, and the numbers of mycobacteria in their organs were determined by quantitative bacteriologic techniques (table 7).

As previously shown for virulent organisms (8, 9, 37), it may be seen in table 7 that the fate of attenuated mycobacteria *in vivo* is markedly affected by the nutritional regimen. The numbers of viable organisms recovered from the spleen and from the lungs were much larger in animals fed the inadequate diet or subjected to fasting than in those fed pellets uninterruptedly. It also may be seen that many more viable organisms were recovered from animals inoculated with RIRv than from those inoculated with BCG-*B*, whatever the nutritional regimen.

TABLE 7

EFFECT OF DIET ON NUMBERS OF VIABLE ORGANISMS RECOVERED FROM SPLEEN AND LUNGS OF MICE INFECTED WITH LARGE DOSES OF RIRv OR BCG-B

Strain Injected (0.2 ml. intravenously)	Nutritional Regimen	Numbers of Colonies* Recovered 8 Weeks After Infection from	
		Spleen	Lungs
RIRv	Pellets ad libitum uninterruptedly	1,300	92,600
RIRv	Pellets with two fasting periods	6,260	>100,000
RIRv	Inadequate diet	>10,000	>100,000
BCG *B*	Pellets ad libitum uninterruptedly	20	3
BCG-*B*	Pellets with two fasting periods	125	20
BCG-*B*	Inadequate diet	135	31

* Each figure represents the average for 4 mice.

TABLE 8

COMPARATIVE FATE OF VARIOUS ATTENUATED MYCOBACTERIA IN THE LUNGS OF MICE MAINTAINED ON TWO NUTRITIONAL REGIMENS

Strain Injected (0.2 ml. intravenously)	Number of Colonies Recovered from Lungs 8 Weeks After Infection	
	Mice Fed Uninterruptedly	Mice Fasted on Two Occasions
BCG-*A*	19,400	26,400
BCG-*B*	3	20
BCG-*C*	1,480	2,100
BCG-*D*	420	797
RIRv	92,600	>100,000

In table 8 the results of a similar experiment are presented in which 0.2 ml. of culture RIRv or of the BCG substrains *A*, *B*, *C*, and *D* was injected into mice, half of which were fed pellets uninterruptedly, and the other half deprived of food for two periods of two days at weekly intervals. In this case again, comparison of the numbers of viable cells recovered from the spleens and lungs suggested that the cultures could be placed in the following descending order of activity *in vivo*: RIRv, BCG-*A*, BCG-*C*, BCG-*D*, and BCG-*B*, irrespective of the nutritional regimen.

The Production of Fatal BCG Infection in Mice

Although the results just described establish that RIRv and BCG can multiply and survive for prolonged periods of time in mice, the infectious processes caused by these attenuated organisms are usually self limited and do not result in a fatal outcome under normal circumstances. On the other hand, it is apparent from the results presented in tables 6, 7, and 8 that the nutritional and physiologic disturbances known to increase the mortality of mice infected with virulent tubercle bacilli (8, 9, 37) also favor the multiplication and survival of attenuated cultures in these animals. Indeed, it will presently be shown that BCG infection can become progressive and bring about death in mice maintained under abnormal physiologic conditions.

Cultures of BCG-*A* and BCG-*B* were grown for eight days in liquid Tween albumin medium. Amounts of 0.2 ml. of these cultures were injected intravenously into albino mice. Half of the animals were fed Sherman diet (660 gm. of whole wheat flour, 330 gm. of dried whole milk (Klim®), 10 gm. of NaCl); the other half received the low protein, high carbohydrate, high citrate diet previously described. In both cases, the ingredients of the diet were resuspended in an equal weight of a solution of 7.5 per cent gelatin, and the mixture was allowed to gel in the refrigerator. The animals were given water and either one of the diets, ad libitum, throughout the period of experimentation. The cumulative numbers of deaths at weekly intervals are recorded in table 9.

As may be seen in table 9, no deaths occurred among the mice infected with BCG-*A* or BCG-*B* and fed the Sherman diet, whereas all animals eventually died in the groups fed the low protein, high carbohydrate, citrate diet. Of the animals which had received BCG-*A*, 50 per cent were dead within six weeks after infection; in contrast, deaths began to occur only after the seventh week among animals infected with BCG-*B*.

TABLE 9

FATAL COURSE OF BCG INFECTION IN MICE FED AN INADEQUATE DIET

BCG Strain (0.2 ml. intravenously)	Diet	Cumulative Numbers of Deaths* at Indicated Times After Infection				Total Deaths (of 10)
		6 weeks	7 weeks	8 weeks	9 weeks	
A	Inadequate†	5	8	10	10	10
A	Sherman	0	0	0	0	0
B	Inadequate†	0	2	3	10	10
B	Sherman	0	0	0	0	0

* Of 10 mice.

† This diet was low in protein, high in carbohydrate and citrate (*see* text page 689).

TABLE 10

DELAYED DEATHS OF MICE INFECTED WITH LARGE DOSES OF BCG

BCG Injected (0.2 ml. intravenously)	Diet ad Libitum	Cumulative Numbers of Deaths* at Indicated Times After Infection				Total Deaths (of 10)
		1 month	2 months	3 months	4 months	
A	Sherman	0	2	3	5	5
A	Pellets	0	0	0	0	0
B	Sherman	0	0	0	1	1
B	Pellets	0	0	0	0	0
C	Sherman	0	1	2	4	4
C	Pellets	0	0	0	0	0
D	Sherman	0	0	0	0	0
D	Pellets	0	0	0	0	0

* Of 10 mice.

In another experiment, albino mice were infected intravenously with 0.2 ml. of BCG cultures of the *A*, *B*, *C*, and *D* substrains grown for ten days in liquid Tween-albumin medium. Half the animals in each group were fed Sherman diet; the other half, pellets. The cumulative numbers of deaths are recorded in table 10.

As may be seen in table 10, none of the mice which were fed pellets had died of BCG infection within the four months of observation, whereas 5 of 10 animals which were fed the Sherman diet died of infection with BCG-*A*, 4 with BCG-*C*, and one with BCG-*B*.

Further evidence that nutritional and physiologic stresses can render mice highly susceptible to certain substrains of BCG is presented in the following experiment.

Mice were infected intravenously with 0.2 ml. of an eight-day-old culture of either BCG-*A* or BCG-*B* in liquid Tween-albumin medium. They were fed ad libitum pellets or one of three diets of the following composition:

	Basic	Cocoa Butter	Thyroid Extract
White flour	650 gm.	450 gm.	650 gm.
Skim milk (dry)	340 gm.	40 gm.	340 gm.
Cerelose	0	300 gm.	0
Salt mixture[3]	10 gm.	10 gm.	10 gm.
Cocoa butter	—	200 gm.	—
Thyroid extract (USP-powdered)[4]	—	—	600 mg. (added to salt mixture)

The above ingredients, with the exception of the cocoa butter, were mixed thoroughly by sifting. One thousand milliliters of a 7.5 per cent gelatin solution in hot water was added to the basic and to the thyroid diets while stirring rapidly. The cocoa butter was emulsified in the hot gelatin solution before adding to the mixture.

The cumulative numbers of deaths at weekly intervals are recorded in table 11.

As may be seen in table 11, there was no mortality among the infected mice fed pellets ad libitum. In contrast, the mortality rate was high among mice infected with BCG-*A* which were either treated with an excess of thyroid extract or fed the inadequate cocoa butter diet. The fact that hardly any of the mice infected with BCG-*B* died when subjected to these abnormal regimens constitutes further evidence of the high degree of attenuation of this substrain.

Comparative Susceptibility of Strains of Mice to BCG Infection

The preceding experiments have made clear that the behavior of the various substrains of BCG in mice is profoundly affected by the nutritional and physiologic state of the infected animals. It will now be shown that the genetic constitution of the animals is also a decisive factor in their response to BCG infection.

Three different strains of mice were used in this experiment: (*a*) the so-called Rockefeller Swiss albino; (*b*) C57 black; (*c*) dba (line 2). The origin of these strains has been described in an earlier publication from this laboratory (32). It was shown that they differ markedly in their resistance to virulent tuberculous infection; the albino mice are the most resistant and the dba mice the least. In the present experiment, animals were infected intravenously with 0.2 ml. of a seven-day-old culture of BCG-*A* in Tween-albumin medium. Half of them were fed the Sherman diet, the other half an inadequate corn meal diet (described in reference (14)). The cumulative numbers of deaths at weekly intervals are recorded in table 12.

Among infected albino mice (so-called Rockefeller Swiss strain), only one of 8 died during the period of observation, irrespective of the composition of the diet. In contrast, the mortality was high in all of the other groups, particularly among animals fed the corn meal diet. On this nutritional regimen, 6 of the 8 C57 mice and all of the dba mice died of the BCG-*A* infection within four weeks. Thus it is apparent that the genetic constitution of the animals and the nutri-

[3] *See* composition of salt mixture, footnote on page 689.

[4] The total daily intake of thyroid extract per mouse was estimated to be 1.5 mg. on the basis of consumption of approximately 5 gm. of food per day.

TABLE 11

COMPARATIVE ABILITY OF TWO SUBSTRAINS OF BCG TO CAUSE FATAL DISEASE IN MICE SUBJECTED TO NUTRITIONAL OR PHYSIOLOGIC STRESSES

BCG Strain (0.2 ml culture)	Regimen ad Libitum	Cumulative Numbers of Deaths* at Indicated Times After Infections						Total Deaths (of 10)
		5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	
A	Excess thyroid	4	7	8	8	8	8	8
B	Excess thyroid	0	1	2	2	2	2	2
A	Inadequate diet	0	1	2	3	4	6	6
B	Inadequate diet	0	0	0	0	0	0	0
A	Synthetic diet	1	0	0	0	2	2	2
B	Synthetic diet	1	0	2	2	2	2	2
A	Pellets	0	0	0	0	0	0	0
B	Pellets	0	0	0	0	0	0	0

* Of 10 mice.

TABLE 12

EFFECT OF MOUSE STRAIN AND DIET ON MORTALITY CAUSED BY BCG INFECTION

Mouse Strain	Diet Fed ad Libitum	Cumulated Numbers of Deaths* at Indicated Times After Infection				Total Deaths (of 8)
		1 week	2 weeks	3 weeks	4 weeks	
Albino	Corn meal	0	0	0	1	1
Albino	Sherman	0	0	0	1	1
C57 black	Corn meal	1	4	4	6	6
C57 black	Sherman	1	1	2	2	2
dba (line 2)	Corn meal	0	2	3	8	8
dba (line 2)	Sherman	1	3	4	4	4

* Of 8 mice.

tional regimen on which they are maintained affect in the same manner susceptibility to infection with BCG and with virulent tubercle bacilli.

Identical Behavior of Two Independent Preparations of BCG Vaccine

The purpose of the present study has been to demonstrate that profound inherent differences exist among the various substrains of BCG in their ability to multiply and to survive *in vivo*. It is well known, on the other hand, that the techniques used for the preparation and preservation of the bacillary suspensions differ from one vaccine-producing laboratory to another and thus contribute still further to the lack of uniformity of the finished product. Emphasis on these difficulties does not imply, however, a nihilistic attitude toward the problem of standardization of the vaccine. Indeed, it seems appropriate on this account to present one last example taken from the experience in this laboratory. This example concerns two vaccine preparations obtained from two independent

TABLE 13

COMPARISON OF TWO FLUID PREPARATIONS OF VACCINE IN VITRO AND IN VIVO

	Numbers of Colonies Recovered from Vaccine Preparations*							
BCG-*G*	(17	20	22	23)	(19	22	24	25)
BCG-*H*	(9	10	11	14)	(11	12	13	13)

	Dose Injected per Mouse (intravenously)	Numbers of Colonies Recovered per Individual Spleen 2 Weeks After Infection†		
BCG-*G*	0.2×10^{-3} ml.	620	655	720
BCG-*H*	0.2×10^{-3} ml.	600	620	640

* Two separate samples of each vaccine were diluted to 1:100,000; four platings of each dilution were made. The figures indicate the numbers of colonies yielded by 0.025 ml. of the final dilution of each sample.

† Figures to be multiplied by 100 to give total numbers of colonies per individual spleen.

institutes, far apart one from the other, which proved undistinguishable when tested by the techniques reported in the present paper.

Immediately upon receipt from the producing laboratories, the preparations of fluid vaccines *G* and *H* (as distributed for use in man) were stored for one week at 4°C. Studies were then carried out to determine (*a*) the numbers of viable units that they contained and (*b*) their behavior in mice.

Two samples of each vaccine were taken and diluted separately. The 1:100,000 dilution of each sample was plated in quadruplicate on oleic-acid–albumin agar (upper part of table 13).

Mice were injected by the intravenous route with 0.2 ml. of 1:1,000 dilution of vaccine and were sacrificed two weeks later. The numbers of colonies recovered on oleic acid–albumin agar from the spleen are presented in the lower part of table 13.

As seen in the upper part of table 13, the numbers of viable units yielded by vaccine *G* were of the same order as those yielded by vaccine *H*. The behavior of the two vaccines in mice was also identical, at least as far as could be judged from the numbers of colonies recovered from the spleen two weeks after infection (lower part of table 13). It is worth noting, furthermore, that the morphologic appearance of the colonies yielded by the two strains on oleic-acid–albumin agar was undistinguishable, being similar to that shown in figure 2*a* in the preceding paper of this series.

SUMMARY

A comparative study has been made of the behavior in experimental animals of several substrains of BCG, as well as of the attenuated human strain RHRv. These organisms have been tested either in the form of subcultures in liquid Tween®-albumin medium or of vaccine preparations obtained from the producing laboratories and ready for use in human beings.

The comparative tests have been based on the use of graded dilutions of vaccines or of young cultures of the same age. This technique has made it possible

to refer biologic activity *in vivo* to known numbers of bacterial cells in a comparable physiologic state.

The tests in guinea pigs have consisted in the measurement of the severity and duration of the skin lesions produced by the intradermal injection of culture dilutions ranging from 1:10 to 1:300. It was found that the BCG substrains tested could be arranged in a consistent order of activity by comparing in time the pathologic effects produced by each culture dilution. In terms of dilutions producing the same pathologic effects, the activity ratios among cultures spread over a range of thirty-fold.

Reproducible and profound differences among attenuated mycobacteria could also be brought out by injecting graded amounts of cultures intravenously into mice.

In all cases RIRv gave rise to higher population levels in the various organs and persisted longer in a viable form than did any of the BCG tested. The BCG substrains differed profoundly among themselves in terms of these two criteria. They could be arranged in a reproducible order of relative attenuation of virulence on the basis of the numbers of colonies recovered from homogenates of the organs at specified times following intravenous injection of standard amounts of culture.

It was possible to increase the severity of the infectious processes caused by RIRv or BCG by subjecting the test mice to metabolic or nutritional disturbances known to increase the susceptibility of these animals to other bacterial infections. It was found, moreover, that the genetic lines of mice most susceptible to virulent tubercle bacilli were also most susceptible to BCG infection.

These observations have made it possible to design experiments in which a high percentage of mice died of infection with RIRv and BCG. The order of relative virulence revealed by these fatal infections (in mice either of high genetic susceptibility or subjected to metabolic disturbances) was the same as that brought out by quantitative studies of the bacterial population reached in the organs of normal resistant mice.

It is concluded that the various substrains of BCG can be arranged in a reproducible order of relative attenuation by a variety of tests in guinea pigs and in mice. The tests are designed to follow quantitatively the fate of the bacteria *in vivo* and the severity of the pathologic processes to which they give rise in animals either normal or under stress.

SUMARIO

Caracteristicas Diferenciales in Vitro e In vivo de Varias Subcepas del BCG: III. Multiplicación y Sobrevivencia In Vivo

Este estudio comparado versó sobre el comportamiento en los animales de experimentación de varias subcepas del BCG, e igualmente de la cepa humana atenuada RIRv. Estos microbios fueron comprobados ya en forma de subcultivos en medio líquido de Tween®-albúmina o de preparaciones vacunales obtenidas de los laboratorios productores y dispuestas para empleo en seres humanos.

Las pruebas comparativas se basaron en el uso de diluciones graduadas de vacunas o de cultivos jóvenes de la misma edad. Esta técnica permitió correlacionar la actividad

biológica *in vivo* con cantidades conocidas de células bacterianas en estado fisiológico comparable.

Las pruebas en cobayos consistieron en la medición de la gravedad y la duración de las lesiones cutáneas producidas por la inyección intradérmica de diluciones de cultivos, que variaban de 1:10 a 1:300. Observóse que podían colocarse las subcepas de BCG comprobadas en un orden compatible de actividad comparando cronológicamente los efectos patológicos producidos por cada dilución del cultivo. En términos de diluciones que producían los mismos efectos patológicos, las proporciones de la actividad entre los cultivos abarcaban una escala cuyo extremo superior era cincuenta veces mayor que el inferior.

Pudieron también provocarse diferencias reproducibles y profundas entre las micobacterias atenuadas inyectando cantidades graduadas de cultivos intravenosamente en los ratones.

En todos los casos, RIRv dió origen a niveles más altos de población en los varios órganos y persistió más tiempo en forma viable que ninguno de los BCG ensayados. Las subcepas de BCG discreparon intensamente entre sí a base de esas dos pautas, siendo posible colocarlas en un orden reproducible de relativa atenuación de la virulencia a base del número de colonias obtenidas de los homogeneatos de los órganos en momentos estipulados después de la inyección intravenosa de cantidades fijas de cultivo.

Resultó posible acrecentar la gravedad de los procesos infecciosos ocasionados por RIRv o BCG sometiendo los ratones de ensayo a trastornos metabólicos o nutritivos que se sabe acrecientan la susceptibilidad de esos animales a otras infecciones bacterianas. Observóse, además, que las estirpes genéticas de los ratones más susceptibles a bacilos tuberculosos virulentos fueron también las más susceptibles a infección por BCG.

Estas observaciones han permitido proyectar experimentos en los que un elevado porcentaje de ratones murió de infección por RIRv o BCG. El orden de relativa virulencia revelado por estas infecciones letales (en ratones ya de alta susceptibilidad genética o sometidos a trastornos metabólicos) fué idéntico al mostrado por extudios cuantitativos de la población bacteriana alcanzada en los órganos de ratones resistentes normales.

En conclusión: cabe disponer las varias subcepas de BCG en un orden reproducible de relativa atenuación mediante varios ensayos en cobayos y en ratones—ensayos estos destinados a observar cuantitativamente el destino de las bacterias *in vivo* y la gravedad de los procesos patológicos que producen en animales ya normales o bajo tensión.

RESUME

Caractères différentiels in vitro et in vivo de diverses souches dérivées du B.C.G.: III. Multiplication et survie in vivo

Une étude comparative a été faite du comportement *in vivo* de plusieurs sous-souches dérivées du BCG et de la souche humaine atténuée RIRV. Ces germes ont été testés soit sous la forme de sous-cultures dans un milieu liquide Tween-albumine, soit sous la forme de préparations de vaccins obtenues de laboratoires de production et destinées à l'utilization chez l'homme.

Les tests de comparaison ont été fondés sur l'emploi de dilutions de vaccin ou de jeunes cultures. Cette technique a permis de comparer l'activité biologique *in vivo* de germes dans un état physiologique comparable.

Chez le cobaye, les tests ont consisté à déterminer la sévérité et la durée des lésions cutanées provoquées par l'injection intradermique de dilutions de cultures variant entre 1/10 et 1/300. Il a été observé que les sous-souches dérivées du BCG soumises à ces tests pouvaient être classifiées selon un ordre d'activité constante en comparant les effets pathologiques obtenus avec chaque dilution par rapport au temps. Les activités de ces sous-souches exprimées en dilutions produisant les mêmes effets pathologiques ont présenté des différences de l'ordre de 1 à 30.

Des différences profondes et reproductibles entre les mycobactéries atténuées ont été

aussi mises en évidence en injectant des quantités déterminées de cultures par voie intraveineuse chez la souris. Dans tous les cas, RIRV a donné lieu à une population microbienne plus élevé dans les divers organes, et a persisté plus longtemps qu'aucun des BCG testés. Les sous-souches dérivées du BCG ont différé entre elles du point de vue de ces deux critères.

En comparant les nombres de colonies obtenus à partir d'homogénats d'organes, à des intervalles donnés après l'injection intraveineuse d'une quantité standard de culture, il a été possible de classifier ces souches du point de vue de l'atténuation relative de leur virulence.

Il a été possible d'accroître la sévérité du processus infectieux causé par RIRV ou BCG en soumettant des souris à des troubles métaboliques ou nutritionels aptes à accroître la sensibilité de ces animaux à d'autres infections bactériennes. En outre, il a été observé que les lignées génétiques de souris les plus sensibles aux bacilles tuberculeux virulents sont également les plus sensibles à l'infection par le BCG.

Ces observations ont permis de procéder à des expériences dans lesquelles un pourcentage élevé de souris ont succombé à la suite de l'infection par RIRV ou BCG. L'ordre de virulence relative révélé par ces infections mortelles (chez des souris de sensibilité génétique élevée ou soumises à des troubles métaboliques) s'est trouvé être le même que celui révélé par des études quantitatives de population bactérienne dans les organes de souris de résistance normale.

En conclusion: Les sous-souches dérivées du BCG peuvent être classifiées en ordre d'atténuation relative de leur virulence, par divers tests chez le cobaye et la souris—tests qui permettent de déterminer quantitativement le sort des bactéries *in vivo* et la sévérité des processus pathologiques qu'elles causent chez les animaux normaux ou soumis à des conditions de stress.

(The relevant bibliography will be found at the end of the fourth paper of this series.)

DIFFERENTIAL CHARACTERISTICS IN VITRO AND IN VIVO OF SEVERAL SUBSTRAINS OF BCG[1]

IV. Immunizing Effectiveness

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INTRODUCTION

It has been shown in the preceding papers of this series that the various substrains of BCG differ in their growth requirements *in vitro*; their morphologic characteristics *in vitro* and *in vivo*; and their ability to multiply, to survive, and to cause disease in experimental animals. Experiments published elsewhere have provided evidence that the time of appearance of immunity, and the duration of the immune state after BCG vaccination, are conditioned by the rate of multiplication and of disappearance of the BCG bacilli in the vaccinated individual. The present paper deals primarily with the relation between development of the immune state and genetic endowment of the vaccinating bacilli. It also presents new evidence that the immunity elicited by BCG vaccination is an expression of the ability of the living attenuated bacilli to multiply *in vivo*.

OBSERVATIONS

Three different criteria have been used to demonstrate and evaluate the ability of BCG vaccination to increase the resistance of experimental animals to tuberculosis: the survival time of the animals following virulent challenge infection; the fate of the virulent bacteria in their organs; and the extent and histologic characteristics of the lesions caused by the challenge infection. These criteria can be and have been applied to many different animal species. The present paper deals only with the comparative ability of various substrains of BCG to retard the multiplication of virulent bacilli in the organs of albino mice and to increase the survival time of these animals after challenge infection.

The first experiment to be considered was designed to compare the effect that vaccination with equal amounts of culture of either one of two different BCG substrains has on the multiplication of virulent bacilli in the spleens of mice injected at various times after administration of the vaccine. The materials and procedure used in this particular experiment, as well as the results, have been described in detail elsewhere (15). The findings are again presented here in a new schematic form (figure 1); it seems useful, therefore, to outline briefly the design of the tests to which they refer.

Mice were vaccinated by the intravenous injection of 0.2 ml. of culture dilutions (in 0.1 per cent albumin) of BCG-*A* or BCG-*B*. Control animals received 0.2 ml. of the same diluent. It was shown by plate counts that the volume of culture dilutions injected contained 6,400 viable units of BCG-*A*, or 5,600 viable units of BCG-*B*. In order to follow the

[1] From the laboratories of The Rockefeller Institute for Medical Research, New York, New York.

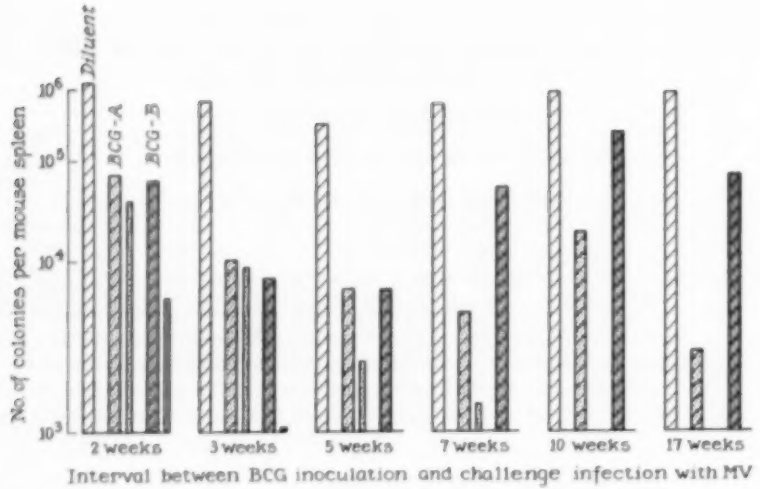

FIG. 1. Recovery of virulent tubercle bacilli (MV) from mice infected at various intervals after vaccination with living BCG-*A* or BCG-*B*.

Numbers of colonies of virulent tubercle bacilli (MV) recovered from mice receiving diluent instead of BCG, and challenged with MV (4 mice per group).

Numbers of colonies of virulent tubercle bacilli (MV) recovered from mice vaccinated with BCG-*A* and challenged with MV (4 mice per group).

Numbers of BCG colonies recovered from mice vaccinated with BCG-*A* but not challenged (3 mice per group).

Numbers of colonies of virulent tubercle bacilli (MV) recovered from mice vaccinated with BCG-*B* and challenged with MV (4 mice per group).

Numbers of BCG colonies recovered from mice vaccinated with BCG-*B* but not challenged (3 mice per group).

fate of the injected bacilli in the mouse tissues, 3 mice of each vaccinated group were sacrificed two, three, five, seven, ten, and seventeen weeks after vaccination; the size of the bacterial population in the spleen was determined by colonial enumeration on albumin agar. At the same times, 4 mice of each vaccinated group and 4 of the control group were infected intravenously with 0.2×10^{-8} ml. of virulent culture MV. These animals were sacrificed two weeks after the challenge infection in order to determine the numbers of living bacilli in their spleens. The composite results of this experiment are presented in figure 1.

In the groups challenged with virulent bacilli two weeks after vaccination with BCG, approximately the same numbers of colonies were obtained whether BCG-*A* or BCG-*B* had been used as vaccinating agent.[2] These numbers were of

[2] During the periods when BCG-*A* organisms were recovered from mice which had not received the challenge virulent infection, the colonies recovered from challenged animals included both those of the virulent and of the BCG strains since these two cannot be differentiated on the basis of colonial morphology (*see* figures 1 and 3 in the second paper of this series).

the order of one-tenth of that found in the nonvaccinated animals, indicating a high degree of immunity. Evidence of immunity became even more striking in vaccinated mice challenged three or five weeks after vaccination, the difference in numbers of colonies yielded by the vaccinated and nonvaccinated groups being then of the order of one hundred fold—again irrespective of the BCG substrain used for vaccination.

Challenge infections carried out seven, ten, and seventeen weeks after infection revealed a progressive loss of immunity in mice vaccinated with BCG-*B*, but not in those vaccinated with BCG-*A*.

It is important to note that the BCG-*B* organisms had rapidly disappeared from the mouse organs, no colony being recovered from the spleen of the vaccinated (but not challenged) animals three weeks after vaccination. In contrast, the BCG-*A* organisms persisted much longer in the organs. It is likely, therefore, that the progressive loss of immunity in mice vaccinated with BCG-*B* was a consequence of the rapid disappearance of these bacilli from the mouse tissues. This relation between persistence of BCG and duration of immunity is further illustrated in the following experiment, in which the challenge infection took place at long periods of time (twelve, twenty-four, or thirty-two weeks) after vaccination.

Mice were vaccinated by the intravenous injection of various amounts of cultures of BCG-*A* or BCG-*B* diluted in a final volume of 0.2 ml. of 0.1 per cent bovine albumin. The dose administered corresponded for BCG-*A* to 250,000, or 250 viable units, and for BCG-*B* to 180,000, or 1,800 viable units. The unvaccinated control animals received 0.2 ml. of diluent. Four animals of each vaccinated group were sacrificed twelve, twenty-four, and

TABLE 1

EFFECT OF BCG SUBSTRAIN, DOSE OF VACCINE, AND TIME OF VACCINATION ON NUMBERS OF BCG AND OF VIRULENT COLONIES RECOVERED FROM SPLEENS OF MICE

Vaccination		Time Between Vaccination and Challenge	Numbers of Colonies* Recovered from Individual Spleens 2 Weeks After Infection							
			BCG				Virulent Bacilli			
BCG Substrain	Viable units Injected	*weeks*								
A	2.5×10^5	12	5	4	24	32	3	23	176	146
A	2.5×10^5	24	0	0	0	1	26	130	240	—
A	2.5×10^5	32	0	0	0	0	7	10	108	200
A	2.5×10^3	12	1	0	0	0	13	25	58	116
A	2.5×10^3	24	5	0	0	0	180	210	460	—
A	2.5×10^3	32	0	0	0	0	95	119	—	—
B	1.8×10^5	12	0	0	0	0	9	20	57	58
B	1.8×10^5	24	0	0	0	0	90	145	200	210
B	1.8×10^5	32	0	0	0	0	210	310	420	450
B	1.8×10^3	12	0	0	0	0	107	113	129	134
B	1.8×10^3	24	0	0	0	0	125	320	—	—
B	1.8×10^3	32	0	0	0	0	370	490	730	1,040
Controls (diluent)		12					760	1,030	1,450	1,610
Controls (diluent)		24					2,200	2,400	—	—
Controls (diluent)		32					4,500	8,300	11,200	—

* To be multiplied by 100 for whole organ.

thirty-two weeks after vaccination in order to determine the numbers of viable units of BCG-*A* or BCG-*B* present in their spleens. At the same time 4 mice of each vaccinated group and 4 of the control group were infected intravenously with 0.2×10^{-3} ml. of the virulent bovine culture MV (diluted in 0.1 per cent rabbit serum). These animals were sacrificed sixteen days after the challenge infection and the numbers of organisms in their spleens were determined as usual (table 1).

As was to be expected from the results of the preceding experiment (figure 1), no colony of BCG-*B* could be recovered twelve, twenty-four or thirty-two weeks after vaccination, even from animals receiving the largest vaccinating dose (1.8×10^8 viable units) (table 1). In contrast, several of the animals vaccinated with BCG-*A* yielded colonies of this organism, thus confirming that BCG-*A* can persist much longer than BCG-*B* in the organs of mice. Similarly, it is apparent that immunity was beginning to wane thirty-two weeks after vaccination in mice which had received BCG-*B*, whereas it was still at its maximal level at that time in the case of BCG-*A*.

In the two preceding experiments, the effect of vaccination was tested in mice sacrificed a short time after having received the virulent infection. As can be seen in table 2, the difference in duration of the immunity elicited by BCG substrains *A* and *B* was also manifest when sacrifice of the challenged animals took place twenty-eight weeks after infection.

Mice were vaccinated by the intravenous injection of either BCG-*A* (80,000 viable units) or BCG-*B* (220,000 viable units). Six weeks after vaccination, 8 animals of each vaccinated group (and 8 animals of the unvaccinated control group) were infected intravenously with 50,000 viable units of the virulent bovine culture MV. Half of the animals were sacrificed three weeks and the other half twenty-eight weeks after challenge infection. A similar series of tests was carried out on animals which received the challenge infection sixteen weeks after vaccination. Mice which received the vaccinating dose of BCG-*A* or BCG-*B*, but no virulent bacilli, were also sacrificed at the same time as those which received the challenge infection. In table 2 are presented the numbers of colonies of BCG organisms and of virulent bacilli recovered from the spleens of sacrificed animals.

In agreement with the findings presented in figure 1 and table 1, those in table 2 show that BCG-*A* persisted longer in the spleens of mice than did BCG-*B*. Similarly, the immunity elicited by the former strain appeared more effective, particularly when the sacrifice of the animals which received the virulent bacilli was delayed a long time (twenty-eight weeks) after infection.

The differences seen in figure 1, table 1, and table 2 between the immunity elicited by the substrains *A* and *B* of BCG appear to be related to differences in the behavior of these organisms *in vivo*. In general, BCG-*B* does not reach as high population levels in the organs, or persist as long, as does BCG-*A*. This difference of behavior *in vivo* holds true even when much larger doses of BCG-*B* than of BCG-*A* (a thousand fold or more) are injected into mice. On the other hand, many experiments carried out in this laboratory have revealed that BCG-*B* can produce immunity as rapidly as BCG-*A* if injected under conditions that permit a rapid accumulation of bacillary protoplasm in the vaccinated individual. One experiment will suffice to illustrate this phenomenon.

Groups of 8 mice were vaccinated by the intravenous injection of graded doses of BCG-*A*

TABLE 2

EFFECT OF BCG SUBSTRAIN, DOSE OF VACCINE, TIME OF CHALLENGE AFTER VACCINATION, AND TIME OF SACRIFICE AFTER CHALLENGE ON NUMBERS OF BCG AND VIRULENT BACILLI RECOVERED FROM SPLEENS OF MICE

Vaccination		Time Between Vaccination and Challenge	Time Between Challenge and Sacrifice	Numbers of Colonies* Recovered from Spleens of Individual Mice									
BCG Substrain	Viable Units Injected			BCG				Average	Virulent Bacilli				Average
		weeks	weeks										
A	8.0×10^7	6	3	150	360	—	—	260	50	50	50	170	80
A	8.0×10^6	6	28	20	30	80	180	80	130	350	—	—	240
B	2.2×10^8	6	3	1	4	9	—	5	160	420	550	1,110	560
B	2.2×10^6	6	28	0	0	0	0	0	90	340	590	1,090	530
Nonvaccinated controls		6	3						2,900	3,900	4,000	4,400	3,800
Nonvaccinated controls		6	28						280	1,150	2,050	1,150	2,400
A	8.0×10^7	16	3	2	14	25	260	80	70	230	550	640	370
A	8.0×10^6	16	28	0	0	4	30	9	30	90	160	400	170
B	2.2×10^8	16	3	1	1	10	—	4	310	360	760	1,000	610
B	2.2×10^6	16	28	0	0	0	0	0	120	1,050	3,800	5,600	2,640
Nonvaccinated controls		16	3						3,200	4,500	5,000	5,500	4,550
Nonvaccinated controls		16	28						3,100	4,000	5,500	—	4,200

* To be multiplied by 100 for whole organ.

or BCG *B* diluted in a final volume of 0.2 ml. of 0.1 per cent bovine albumin. The doses injected corresponded to 10^8 or 10^6 viable units for BCG-*A* and 9×10^8 or 9×10^6 viable units for BCG-*B*. Three subgroups of mice were used for each dose of BCG; one subgroup was vaccinated two days before challenge infection; the second, one week; and the third, two weeks before. The challenge infective dose consisted of 0.2 ml. of an eight-day-old culture of the virulent bovine strain MV in Tween®-rabbit serum medium. The time of death for individual mice and the cumulative numbers of deaths at weekly intervals are presented in table 3.

As may be seen in table 3, evidence of protection could be detected in mice which had received very large doses of BCG even when this dose had been administered as shortly as two days before challenge infection. This rapid development of immunity occurred just as well in the case of BCG-*B* as of BCG-*A*. With the smaller doses of vaccine, the protective effect became apparent only when virulent infection was postponed for one or two weeks. In the light of earlier results from this laboratory, this delay can be traced to the time required for the BCG bacilli to reach *in vivo* a population level sufficiently high to exert an adequate protective stimulus. It may be noted in passing that, in the present experiment, the time which elapsed between vaccination and challenge infection was not long enough to reveal a difference in the duration of the immunity elicited from the two substrains of BCG.

The view that the establishment and maintenance of immunity elicited by

BCG vaccination (as usually practiced) is an expression of the extent to which BCG multiplies and survives *in vivo* and, consequently, of the amount of antigenic material that accumulates in the tissues, has recently been criticized on the basis that immunity develops even when animals are treated during vaccination with doses of isoniazid sufficient to inhibit bacillary multiplication (2). In the experiments quoted to justify this criticism, mice were vaccinated intraperitoneally with "0.1 ml. of five-day Tween-albumin cultures of BCG." It will be shown in the following experiment that isoniazid treatment does indeed allow the development of immunity when large doses of BCG cultures are used for vaccination, but that the drug interferes with the immune process when the amount of vaccine is smaller, of the order of that used under practical circumstances. It will be shown also that, in animals receiving isoniazid, immunity develops just as well whether killed bacilli or a large dose of living organisms are used as vaccinating material.

Albino mice, three weeks old, were vaccinated by the intraperitoneal injection of either one of the following materials: (*a*) 0.1 ml. of a seven-day-old culture of BCG-*A* in Tween-albumin medium; (*b*) 0.0001 ml. of the same culture diluted in 0.1 ml. of this medium; (*c*) 1 mg. (dry weight) of bacilli from the same culture killed with 2 per cent phenol and washed with acetone; this bacillary suspension was injected in 0.1 ml. of sterile Tween-albumin medium. The unvaccinated controls received 0.1 ml. of sterile medium. All animals were fed pellets and received water ad libitum throughout the course of experimentation. Half of the animals in each group received isoniazid for three days before vaccination and eight

TABLE 3

EFFECT OF BCG SUBSTRAIN, DOSE OF VACCINE, AND VACCINATION TIME ON SURVIVAL OF MICE CHALLENGED WITH LARGE DOSE OF VIRULENT CULTURE

Vaccination		Time Between Vaccination and Challenge	Times of Deaths for Individual Mice in Numbers of Days After Infection								Cumulative Numbers of Deaths at Indicated Times After Infection		
BCG Substrain	Viable Units Injected										1 week	2 weeks	3 weeks
A	1×10^8	2 days	15	15	15	18	18	19	21	21	0	0	8
A	1×10^8	1 week	12	18	20	21	22	22	22	27	0	1	4
A	1×10^8	2 weeks	18	19	19	21	21	21	25	25	0	0	6
A	1×10^6	2 days	12	12	12	15	15	16	16	16	0	3	8
A	1×10^6	1 week	14	14	16	16	16	16	16	18	0	2	8
A	1×10^6	2 weeks	15	18	19	22	25	25	25	*	0	0	3
B	9×10^7	2 days	14	16	19	19	19	19	19	19	0	1	8
B	9×10^7	1 week	19	20	21	21	22	22	25	25	0	0	4
B	9×10^7	2 weeks	7	20	20	21	21	25	25	25	1	1	5
B	9×10^5	2 days	11	13	13	14	15	15	15	18	0	4	8
B	9×10^5	1 week	18	18	18	20	21	21	25	28	0	0	6
B	9×10^5	2 weeks	12	18	19	21	25	25	29	32	0	1	4
Unvaccinated controls			11	13	13	14	14	18	18	19	0	5	8

* One mouse missing at end of experiment.

TABLE 4

IMMUNIZING EFFECT OF BCG VACCINATION, WITH OR WITHOUT PROPHYLACTIC CHEMOTHERAPY

Prophylactic Isoniazid	Vaccination (intraperitoneally)	Cumulative Numbers of Deaths at Indicated Times After Challenge Infection*				
		4 weeks	5 weeks	6 weeks	7 weeks	8 weeks
mg. daily						
5	10^{-1} ml. living BCG	2	3	5	9	12
0	10^{-1} ml. living BCG	3	3	4	8	11
5	10^{-4} ml. living BCG	5	8	12	16	18
0	10^{-4} ml. living BCG	3	4	5	8	12
5	1 mg. killed BCG†	3	3	6	9	11
0	1 mg. killed BCG†	2	2	5	11	13
5	0	5	10	13	14	18
0	0	7	9	14	16	19

* 20 mice infected intravenously with 0.2 ml. of virulent culture MV, ten weeks after vaccination; isoniazid discontinued two weeks before challenge infection.

† Young culture of BCG-A killed with 2 per cent phenol; cells washed with acetone.

weeks after; the isoniazid was administered in the drinking water in concentrations sufficient to correspond to an approximate daily intake of 5 mg. per mouse.

All animals were infected ten weeks after vaccination, i.e., two weeks after discontinuance of the drug, by the intravenous injection of 0.2 ml. of an eight-day-old culture of the virulent bovine strain MV (table 4).

The results presented in table 4 make clear that, when a large dose of BCG was used, either in the form of living culture (0.1 ml.) or of dead bacilli (1 mg.), isoniazid chemoprophylaxis did not prevent mice from becoming immune. In contrast, mice which received smaller doses of living BCG (10^{-4} ml.) failed to develop immunity during the period of drug treatment. These findings are in accord with others recently published (15, 24). It was then shown that the development of the immune state which followed vaccination with small doses (0.2×10^{-5} ml.) of the attenuated human strain, RIRv, was retarded or completely prevented by isoniazid treatment of the mice in course of immunization. Taken together the results with BCG and with RIRv indicate that, in mice receiving vaccinating doses of the order of those used for vaccination under practical circumstances, the development of immunity depends upon extensive multiplication of the attenuated organisms *in vivo*. This multiplication is required to make available an amount of bacillary protoplasm sufficient to elicit in the animal an adequate protective response.

GENERAL DISCUSSION[2]

Most of the studies on BCG variation have been focused on the possibility that the culture might recover the virulence of the bovine strain from which it was initially derived. One need only recall in this regard the reports in the 1920's

[2] The Discussion includes the experimental results presented in all four papers of this series.—*The Editor*

and 1930's that colonies of fully virulent forms could be separated from BCG cultures maintained under certain cultural conditions (reviewed in 18, 21, 34, 38). Interestingly enough, this phenomenon does not seem to have been encountered in any laboratory during recent years, despite much work with BCG all over the world since the end of the war. It is not possible therefore, to evaluate at the present time the significance of the early reports of complete recovery of virulence.

On the other hand, there seems to be no doubt that BCG can undergo variations which, even though not resulting in full pathogenicity, nevertheless affect its behavior *in vitro* and *in vivo*. The pioneering studies of Jensen in this field—mentioned in the third paper of the present series—have been of fundamental importance in revealing that authentic BCG cultures may differ quantitatively one from the other in their ability to produce lesions in the skin of guinea pigs (22, 23, 42). Jensen's findings have been confirmed and extended in a few other laboratories. It has been shown furthermore that several independent criteria can be used to establish differences among BCG cultures, namely metabolic reactions, mode of growth in liquid media, colonial characteristics on agar surfaces, ability to multiply and survive in mice, and similar phenomena (9, 25–28, 30–33, 41, 44–46). Indeed, differences have also been recognized in man with respect to the duration of the allergy and to the severity and incidence of the adenopathies produced by vaccination (19, 22, 25, 45).

It has been emphasized, however, that the differences observed among BCG cultures are not necessarily the expression of inherent genetic differences (3, 4, 43). As was to be expected, slight changes in culture media, in method of cultivation, in age of the bacterial cells, in conditions of their preservation, and other slight environmental changes can be shown to alter many characteristics and activities of BCG both *in vitro* and *in vivo*. These are phenotypic manifestations which do not involve the genotype of the organism. An attempt has been made in the present series of studies to observe the cultural characteristics and behavior of BCG under conditions that would minimize these transient effects of the environment, in order to bring out the differences due to the genetic endowment of the bacteria. The results of comparative studies leave no doubt that the BCG cultures now used for the preparation of the vaccine differ one from the other not only by reason of phenotypic phenomena, but even more frequently because of hereditary genotypic traits which can be revealed by adequate tests both in culture media and in experimental animals.

There is nothing surprising, of course, in the fact that the various substrains of BCG differ in many of their characteristics, *in vitro* and *in vivo*. It is well known that all bacterial species continuously undergo mutation and that environmental factors—usually ill defined and beyond control—determine which mutant form comes to predominate in a given culture. BCG originated by mutation from a virulent strain of bovine tubercle bacilli and, like other microorganisms, it continues to give rise to mutant substrains. There is no such thing as a "fixed" microbial form.

The unexpected finding was rather that several of the substrains of BCG re-

tained during cultivation in this laboratory most of the characteristics which they possessed when first received from the vaccine-producing institutions. Some of these substrains have been transferred more than one hundred times during a period of some ten years at the Rockefeller Institute, and yet detailed observations *in vitro* and *in vivo* have failed to reveal any significant change in their characteristics since the time of receipt. The stability of these cultures, rather than the variability of BCG, is the fact that requires explanation.

It is worth recalling in this respect that the liquid Tween-albumin medium which has been used in this laboratory since 1946 for the maintenance of cultures of mycobacteria provides fairly homogeneous conditions of growth. Relevant to the problem also is the writers' practice of inoculating each tube of medium (5 ml.) with a large seeding (0.1 ml.) of young culture. Both the homogeneous character of the growth and this inoculation technique tend to discourage the establishment of the occasional mutant forms and, consequently, result in a maximum of stability of the culture. In contrast, the procedure most commonly used in the past has been to cultivate BCG either on the surface of solid media or in the form of surface pellicles on liquid media. In either case, surface growth provides conditions favorable for the establishment of localized populations derived from mutants emerging at any particular site of the growth. By using for transfer some fragment of surface culture, an area in which the mutant predominates may be unwittingly chosen, thus providing a selective process in what appears to be a routine transfer operation.

It may seem surprising that changes in the character of the bacterial population could have been overlooked during the extensive controls to which BCG is subjected in the course of vaccine production. The failure to detect these changes is probably due to the fact that most of them are of a kind not readily detectable by the usual techniques of control. All of the substrains of BCG used in the present studies are acid fast in staining reaction and exhibit on egg and other classical media the typical slow growth of tubercle bacilli. All of them elicit tuberculin allergy and some measure of antituberculous immunity when injected in the proper amount into animals or into man. None of them are able to cause progressive disease and death in guinea pigs, in rabbits, or in albino mice under ordinary conditions of test. Thus, all of the critera formulated by Calmette and Guérin, and universally accepted as characterizing the BCG vaccine, are satisfied by the cultures that were sent to this laboratory by the various vaccine-producing laboratories. It was only by introducing other methods of testing and supplementary criteria that it was possible to bring out among the various substrains certain important differences not revealed by the usual control techniques.

It was noted, for example, that differences in growth requirements come to light when the various substrains are cultivated on semisynthetic agar media containing activated charcoal. Culture media of this type which yield luxuriant colonies of certain substrains fail to support growth of others unless supplemented by the proper nutritional factors—oleic acid, cholesterol, and ill-defined constituents of certain peptones (20).

Differences in growth requirements constitute only one of the manifestations of metabolic individuality among substrains. Another manifestation is found in the length of their survival under certain experimental conditions. It has been repeatedly observed in this laboratory that certain BCG cultures do not survive as long as others in Tween-albumin medium and are also much more sensitive to various toxic agents. Striking differences in viability could be brought out by exposing the bacteria to physiologic concentrations of sodium lactate in an anaerobic environment. Under these conditions no viable cells of the substrain *B* of BCG could be recovered after a very few days, while BCG-*A* survived much longer. Interestingly enough, all virulent human and bovine strains so far tested remained viable for many weeks in this anaerobic environment, thus proving much more resistant than any of the BCG. These tests may not be without relevance to the behavior of the bacilli *in vivo*, since local anaerobiosis and accumulation of lactate are two characteristics of the inflammatory areas (11, 12).

Observations on colonial morphology made several years ago provided the first evidence in our laboratory that BCG cultures are not all alike. This early conclusion has now been extensively confirmed by systematic studies of the types of colonies yielded by mycobacteria on albumin agar or oleic-acid–albumin agar—two completely transparent media well suited to morphologic observations. In order to investigate the homogeneity of the bacterial population present in the vaccines prepared for use in man, graded dilutions of different samples were plated immediately upon receipt from the manufacturing institutes. In all cases, a mixture of spreading (H37Rv-like) and nonspreading (H37Ra-like) colonies was obtained from the vaccine preparation, but the relative proportion of these two colonial types varied widely from vaccine to vaccine. As we have seen, some BCG substrains yielded almost exclusively the spreading type of colonies; others almost exclusively, the nonspreading type. Moreover, the relative proportion of the two colonial types remained approximately constant when the vaccines were subcultured in liquid Tween-albumin medium, even after very large numbers of transfers. Substrains *A* and *B*, for example, have been maintained in this medium for almost ten years in this laboratory; and yet they still exhibit the same striking dissimilarity of colonial population.

Efforts to isolate subcultures yielding only one of the two colonial types have met with little success. Subcultures of any given colony eventually gave rise again to the two colonial forms in a relative proportion similar to that occurring in the substrain from which the colony had first been isolated. Thus, it appears as if each BCG substrain were defined by a characteristic rate of mutation affecting the trait spreading $\rightleftarrows$ nonspreading type of growth. A similar situation, although applying to other morphologic and cultural traits, has long been known to occur in other bacterial species.

In addition to these observations *in vitro*, many experiments *in vivo* have provided further evidence that the various substrains of BCG differ in important biologic activities. Before considering some of the results bearing on this problem, it is essential to emphasize that comparative studies of behavior *in vivo* must be

based on the use of inocula of the same age and physiologic state and must contain the same numbers of viable cells. From this point of view, young homogeneous cultures in liquid Tween-albumin medium seem to present advantages over the conventional inocula obtained from weighed bacterial masses obtained from surface growths which are ground to provide suspensions of cells. All results to be considered in the present discussion were obtained by using as infective inocula graded dilutions of young cultures in liquid Tween-albumin. The numbers of viable units in these inocula were determined by quantitative enumeration of colonies on albumin agar.

As already mentioned, marked differences among substrains of BCG can be brought out by injecting measured amounts of culture into the skin of guinea pigs. In this test, the response of the animals can be evaluated in terms of three criteria: the rate of appearance of the lesion at the site of injection; its maximal extent and severity; and its rate of disappearance. With any given infective dose, the various strains of mycobacteria can be arranged in the same order of relative pathogenicity, whichever of these three criteria is used for quantitation. Of even greater interest is the fact that the same order of relative pathogenicity holds true when the measurements are carried out by entirely different techniques in another animal species, namely, the mouse.

The fate of BCG injected intravenously into mice can be followed by sacrificing the animals at the proper intervals of time and determining the numbers and characteristics of bacterial colonies that can be recovered from their organs. Naturally, the course of the infectious process is very much affected by the dose of infective inoculum and by the physiologic state of the culture at the time of injection. When these factors are properly controlled, very striking differences come to light in the intrinsic ability of the various substrains of BCG to multiply and survive in the mouse. In fact, the maximal level of bacterial population reached in the lungs and spleen, and the duration of its persistence, were always much greater in the case of certain substrains than of others, even when the infective inoculum contained many more viable cells of the "weaker" culture than of the less attenuated. Let it be emphasized again at this point that none of the BCG substrains used in the present study could be regarded as virulent for *normal* mice since they did not produce fatal disease when the infective dose (by the intravenous route) was as large as 0.2 ml. and contained at least 10^7 viable units.

Although BCG infection was almost always self limited in normal animals, it is worth noting that it often resulted in a fatal outcome if the animals were in an abnormal metabolic state, caused either by periods of fasting, by inadequate diets, or by treatment with the proper amount of dinitrophenol or thyroid extract. Whatever the method used to disturb the animal metabolism, those cultures of BCG which multiplied most extensively in normal mice also caused the larger number of deaths in animals in a disturbed metabolic state. In experiments not reported here, degrees of attenuation of virulence could also be brought out among BCG substrains by the use of mice of different genetic backgrounds. Certain mouse strains (dba, line 2, and C57 black, for example) proved more

susceptible to infection with BCG than did albino strains. Here again the severity of the pathologic process caused in susceptible strains of mice by a given BCG culture was correlated with the severity of its effect in the more resistant animals.

When the results obtained *in vitro* and *in vivo* are considered together, a close correlation appears between the cultural characteristics and their behavior in animals of the eleven substrains of BCG studied in this laboratory. In all instances, the cultures which were the most robust in culture media and which yielded the largest percentage of spreading type of colonies on albumin agar also produced the most severe and lasting lesions in the skin of guinea pigs, multiplied most extensively, survived longest in the organs of normal mice, and killed the largest percentage of animals in a disturbed metabolic state. However, whereas the correlation between *in vitro* characteristics and behavior *in vivo* seems well established, only very tentative suggestions can be made to account for its mechanism. Before discussing this problem, it is necessary to consider first some related experimental facts.

When cultures of BCG were injected into normal albino mice by the intravenous route, it was found that both the spreading and the nonspreading types of colonies could be recovered from the animals for many weeks after injection. Furthermore, the relative proportion of the two colonial types yielded by the various organs was of the same order as that which existed in the culture injected. Under certain conditions, however, and particularly when the animals were sacrificed several months after infection, a change occurred in the relative proportion of the two colonial types. In the lungs in particular, there was a tendency for the spreading colonies to become predominant. Indeed, these spreading forms constituted at times the only colonial type recovered from this organ. Although the analysis of these phenomena has not been pushed far enough to allow a final interpretation, the findings indicated that during the first few days before the appearance of immunity there is no striking difference between the rates at which the spreading and nonspreading bacterial forms are destroyed in, or eliminated from, the body of normal mice. In contrast, it seemed as if the spreading forms were endowed with a much greater ability to multiply *in vivo*, particularly in the lungs.

Earlier studies have already contributed evidence that a correlation exists between the type of colonial morphology yielded by a given mycobacteria and its pathogenicity, but the basis of this correlation is still obscure. It has been claimed that the cellular constituent which determines the serpentine pattern of growth and the spreading colonial morphology is a lipid possessing toxic properties and is more abundant in virulent than in avirulent cultures. This lipid constituent (the so-called cord factor) would thus play a determinant role in the ability of mycobacteria to multiply *in vivo* and to cause pathologic changes. As shown elsewhere, however, proof is still lacking that toxic lipids are really more abundant in virulent than in attenuated and avirulent cultures, or that they play any part in pathogenicity (9, 11). In fact, it has been found in this laboratory that the phenol-killed cells of the BCG substrain which is less able to multiply *in vivo* (substrain *B*) happen to be more toxic for mice than the cells of another BCG

culture (substrain A) endowed with much greater invasive power. The relation of toxicity of lipids to invasiveness of mycobacteria from which they are derived is therefore still *sub judice*.

There does appear to exist, however, some relation between colonial morphology and certain metabolic characteristics which may be of relevance to behavior *in vivo*. Thus, the cultures of BCG which produce spreading colonies on albumin agar grow more rapidly and abundantly on simple synthetic media; furthermore, they seem to survive longer *in vitro*, particularly in environments deficient in oxygen and containing lactic acid. In view of the fact that the biochemical environment created by the inflammatory process is characterized by partial anaerobiosis and high local concentration of lactic acid (11, 12)—conditions inimical to the multiplication and survival of mycobacteria—it seems not unlikely that the metabolic dissimilarity among BCG substrains may account in part for differences in the ease with which they can establish themselves *in vivo*. Needless to say, extensive comparative studies would be required to determine the validity of this hypothesis and its bearing on the correlation between invasiveness and colonial morphology.

It is now necessary to inquire into the relation that behavior *in vivo* has with immunizing effectiveness. Since there is no proof that tuberculin allergy can serve as an index of immunity—and, indeed, much evidence that the two conditions often follow an independent course—the only meaningful measurement of protective effect of vaccination that can be made at the present time is to determine the increase in resistance to a standardized challenge infective dose. In the present experiments, resistance was measured in terms of two independent criteria: (*a*) survival time of mice infected intravenously with a large dose (0.2 ml.) of virulent culture; (*b*) quantitative determination of the numbers of living bacilli that could be recovered from the organs of mice sacrificed at various intervals of time after intravenous injection of a small dose of virulent culture.

All vaccinated animals exhibited increased resistance to virulent infection when the vaccinating dose was larger than 10^{-3} ml. of BCG culture and when the challenge was given within three months after vaccination. Moreover, no difference in protective effect could be recognized under these conditions among the various BCG substrains, whether the level of immunity was measured by survival time of the challenged animals or by the fate of virulent bacilli in their lungs and spleens. In contrast, differences in immunizing effectiveness became evident when the vaccinating dose was reduced to 0.0002 ml. (or less) of culture or when the time between vaccination and challenge was longer than three months. With BCG substrains that multiplied extensively and survived long in the organs of mice, immunity could be established with doses of culture as low as 10^{-6} ml., and the immune state persisted as long as the experiment lasted (one year). With BCG substrains of low invasiveness, on the other hand, the minimal vaccinating dose was of the order of 10^{-3} ml. of culture, and the immunity began to wane some three months after vaccination.

Because of the large numbers of animals and amount of labor involved in these comparative studies, it has not been possible to carry out detailed immunization

tests with more than a few substrains of BCG. Nevertheless, the results obtained warrant the conclusion that differences in immunizing effectiveness can be readily demonstrated provided small enough amounts of vaccine are used and the challenge infection is delayed. These differences are masked if the vaccinating dose is large and immunity is tested soon after vaccination.

It is difficult to relate these findings to the results of human vaccination since, in man, the BCG vaccine is usually injected by the intradermal route and its effects are measured in terms of tuberculin allergy. Nevertheless, some speculation may be justified. The usual amount of vaccine injected intradermally in man is 0.1 mg. or less, a dose corresponding to approximately 0.5 ml. of BCG culture as used in the present study (the cultures in Tween-albumin medium contain 0.2 to 0.3 mg. bacilli dry weight). Converting from human to mouse weight (15 to 20 gm.), it can be calculated that the human dose of BCG vaccine corresponds to an amount ranging from 10^{-4} to 10^{-3} ml. of culture per mouse. With this dose, as has been shown, no measurable immunity develops unless the bacteria are allowed to multiply extensively *in vivo*. The claim that the immune reaction can take place even if bacillary proliferation is prevented by isoniazid therapy rests on experiments in animals receiving doses of vaccine from one hundred to one thousand fold greater than those permissible in human beings. (For a more extensive documentation and discussion of these problems, *see* table 4 of the present paper and references (10) and (16)). Barring these very artificial conditions, there is no doubt that immunity is an expression of the ability of the BCG organisms to multiply in the body of the vaccinated individual. The immunity is dependable and lasting if the BCG culture is endowed with great invasiveness, whereas it is at best weak and transient if the BCG culture has become very attenuated. Granted that these quantitative relationships must be qualified by a number of complex factors, they are of significance just the same for the practice of human vaccination.

It is well known, of course, that the results of vaccination are rather erratic. From one field test to another, marked variations have been observed in the size of the vaccinal lesion and the percentage of adenopathies, in the intensity of the allergy induced and its persistence, in the magnitude or very presence of the protective effect against virulent infection. The literature on these conflicting reports is so extensive that it cannot be reviewed here; nor is it likely to provide convincing information concerning the reasons which account for the irregularity of results. Many contributing factors immediately come to mind. The metabolic state of the individual at the time of vaccination certainly affects the extent of BCG multiplication in the organs and probably influences also the immunologic reaction to the antigenic stimulus. The number of living bacilli and the amount of dead bacterial protoplasm present in the vaccine are obviously important. Evidence is now accumulating, furthermore, that the age of the culture and its physiologic state play a role in determining its ability to multiply *in vivo*. But, even when all these variables are controlled and standardized as far as possible, there still remains to be considered—as shown by the present experimental study—the effect on behavior *in vivo* of some intrinsic, hereditary properties of the

substrains of BCG employed in the preparation of the vaccine. In order to avoid using the word virulence with its unpleasant connotations, it will suffice for the time being to regard these properties as conditioning the degree of invasiveness of the culture.

Since the most invasive substrains of BCG are the most likely to elicit dependable and lasting immunity, it would appear at first sight that they are best suited to the practice of human vaccination. It must be realized on the other hand that the greater invasiveness increases the incidence of adenopathies which—although without dangerous consequences—are likely to disturb the lay public as well as many physicians. In final analysis, therefore, the choice of the optimal substrain of BCG involves a compromise between the requirements of the immunologist for dependable and lasting immunity and the concern of all for innocuousness. In this case the meaning of inocuousness is determined in large part by the susceptibilities of the population involved. Fastidious suburbanites, for example, are more apt to object to a few abcesses and unsightly scars than are people whose rugged life is spent in the tropics, in the deserts, or in city slums. Physicians who have had occasion to compare the effect of smallpox vaccine in different parts of the world know that preparations acceptable in certain countries would be rejected in others as too prone to cause severe lesions. Yet, there is much evidence that the level and duration of immunity to smallpox is inversely related to the degree of attenuation of the virus used to prepare the vaccine.

Thus, the selection of a strain of BCG for vaccination poses the dilemma that the invasiveness of the strain must be sufficiently, but not excessively, attenuated. This dilemma is not peculiar to BCG; it applies probably to most, if not all, living pathogens used for the vaccination of animals and man. Vaccination always involves a calculated risk. Absolute safety in its practice can be bought only at the cost of effectiveness.

SUMMARY

The antituberculous immunity elicited by BCG vaccination is the outcome of the antigenic response to bacillary material having two different origins: (*a*) the bacilli present in the vaccine preparation; (*b*) the bacilli that multiply *in vivo* after injection. The relative importance of these two components of the immune response depends upon the conditions of the test.

If the dose of vaccine injected is very large, as is often the case in animal experimentation, it can provide outright an intense antigenic stimulus and elicit immunity even though the vaccinating bacilli do not have a chance to multiply *in vivo*. This is the case, for example, if the bacilli have been killed before injection or if the animals receive isoniazid therapy during the course of vaccination. Under these circumstances, the immune response appears to be independent of the inherent characteristics of the BCG substrain used in the preparation of the vaccine.

The results are very different when the amount of vaccine injected is smaller—of the order (per body weight) of that used in human vaccination.

With the doses of vaccine usually employed under practical circumstances,

isoniazid chemoprophylaxis interferes with the development of immunity by inhibiting bacterial multiplication and hence preventing the accumulation *in vivo* of an amount of antigenic material sufficient for an adequate protective response.

With small doses of vaccine, marked differences can be recognized in the immunizing effectiveness of the various substrains of BCG. The more efficiently the BCG organisms multiply *in vivo*, the more rapidly they elicit immunity in the vaccinated animal. Furthermore, the longer they persist in the tissues, the more lasting is the immunity to which they give rise.

It can be presumed, on the other hand, that the BCG substrains which are best able to multiply and survive in experimental animals (in other words, which are least attenuated in virulence) are also the ones which are most prone to cause adenopathies and other pathologic manifestations. The choice of a BCG substrain for human vaccination involves therefore a compromise between invasiveness (with related immunogenic effectiveness) of the vaccine and the risk of accidents that might result from its use in man.

SUMARIO

Características Diferenciales In Vitro e In Vivo de Varias Subcepas de BCG: IV. Eficacia Inmunizante

La inmunidad antituberculosa provocada por la vacunación con BCG es resultado de la respuesta antigénica a materias bacilares que reconocen dos procedencias distintas: (*a*) los bacilos presentes en la preparación vacunal; (*b*) los bacilos que proliferan *in vivo* después de la inyección. La relativa importancia de esos dos componentes de la inmunirreacción depende de las condiciones del ensayo.

Si la dosis de vacuna inyectada es muy grande, como acontece a menudo en la experimentación en animales, puede suministrar en el acto un estímulo antigénico y provocar inmunidad, aunque los bacilos vacunantes no tengan oportunidad de multiplicarse *in vivo*. Así sucede, por ejemplo, si se ha matado a los bacilos antes de inyectarlos o si los animales reciben isoniacidoterapia durante el transcurso de la vacunación. En esas circunstancias, la inmunirreacción parece ser independiente de las características inherentes de la cepa de BCG usada en la preparación de la vacuna.

Los resultados son muy distintos cuando la cantidad de vacuna inyectada es más pequeña—del tenor (por peso vivo) usado en la vacunación humana.

Con las dosis de vacuna empleadas habitualmente en la práctica, la quimioprofilaxia con isoniacida obstaculiza la formación de inmunidad por inhibir la multiplicacion bacteriana e impedir por ello la acumulación *in vivo* de una cantidad suficiente de materias antigénicas para una adecuada respuesta protectora.

Con pequeñas dosis de vacuna, pueden reconocerse notables diferencias en la eficacia inmunizante de las varias subcepas de BCG. Mientras más capazmente se multipliquen *in vivo* los BCG, con mayor rapidez provocan inmunidad en el animal vacunado. Además, mientras más tiempo persistan en los tejidos, más duradera es la inmunidad a que dan origen.

En cambio, cabe presumir que las subcepas del BCG más capaces de multiplicarse y de sobrevivir en los animales de experimentación (en otras palabras, cuya virulencia está menos atenuada) son también aquéllas más susceptibles de ocasionar adenopatías y otras manifestaciones patológicas. La selección de una cepa del BCG para vacunación humana impone, por consiguiente, un balanceo entre la facultad invasora (con la correlacionada eficacia inmunógena) de la vacuna y el riesgo que puede provenir del empleo de ésta en el hombre.

RESUME

Caractères différentiels in vitro et in vivo de diverses souches dérivées du BCG: IV. Pouvoir immunisant

L'immunité antituberculeuse produite par la vaccination au BCG résulte de la réponse antigénique à un matériel bacillaire de deux origines différentes: a) les bacilles présents dans le vaccin; b) les bacilles produits par multiplication *in vivo* après l'injection. L'importance relative de ces deux éléments de la réaction d'immunité dépend des conditions du test.

Si la dose de vaccin injectée est très élevée, comme c'est souvent le cas dans l'expérimentation sur l'animal, elle peut provoquer directement un stimulus antigénique intense et entraîner l'immunité, même quand les bacilles vaccinaux ne peuvent se reproduire *in vivo*. C'est par exemple ce qui se produit lorsque les bacilles ont été tués avant l'injection, ou lorsque les animaux sont soumis à l'isoniazidothérapie au cours de la vaccination. Dans ces circonstances, la réaction d'immunité semble indépendante des caractères inhérents à la souche dérivée du BCG utilisée pour la préparation du vaccin.

Les résultats sont très différents quand la dose de vaccin injectée est plus faible—de l'ordre de celle (par Kg de poids) employée pour la vaccination chez l'homme.

Avec les doses de vaccin habituellement utilisées en pratique la chémoprophylaxie par l'isoniazide intervient dans le développement de l'immunité par son pouvoir d'inhiber la reproduction bactérienne, ce qui empêche l'accumulation *in vivo* d'une quantité de matériel antigénique suffisante pour donner lieu à une réaction protectrice.

Avec les doses faibles de vaccin, des différences marquées dans l'efficacité immunisante des diverses sous-souches dérivées du BCG peuvent être observées. Plus la multiplication des bacilles de BCG est abondante *in vivo*, plus l'immunité qu'ils évoquent chez l'animal vacciné est prompte à se manifester. En outre, plus leur persistance dans les tissus est prolongée, plus l'immunité qu'ils confèrent est durable.

D'autre part, il est permis de supposer que les sous-souches dérivées du BCG les plus aptes à se reproduire et à survivre chez l'animal (autrement dit, celles dont la virulence est la moins atténuée) sont aussi celles les plus aptes à provoquer l'adénopathie et autres manifestations pathologiques. Le choix d'une sous-souche de BCG pour la vaccination humaine doit donc être un compromis entre la capacité du vaccin à envahir l'organisme et le risque d'accidents que son emploi peut entraîner chez l'homme.

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THE INTRACELLULAR ACTIVATION OF PYRAZINAMIDE AND NICOTINAMIDE[1]

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INTRODUCTION

Nicotinamide is essentially devoid of tuberculostatic activity *in vitro*, yet it was found by Chorine (1) and by McKenzie and associates (2) to retard the development of tuberculosis in experimental animals. Pyrazinamide, an analogue of nicotinamide, was synthesized by Kushner and his co-workers (3); it also has a low and variable activity against tubercle bacilli *in vitro* (4–7), but its antituberculous activity *in vivo* is greater than that of nicotinamide (3).

Reports on pyrazinamide therapy in experimental tuberculous infection are to some extent conflicting. It appears, however, to be more effective in the mouse than in the guinea pig. Thus, Malone and associates (8) found a degree of activity in the mouse intermediate between that of streptomycin and PAS, a finding similar to that reported by Solotorovsky and his associates (6). On the other hand, Dessau and others (5) could demonstrate only a moderate degree of activity against tuberculosis in the guinea pig. Steenken and Wolinsky (7) were unable in their first study to demonstrate any significant activity against tuberculosis in this host species. Their later observations with larger doses (7*a*), however, showed that the response in the guinea pig was essentially similar to that seen in the mouse. In man pyrazinamide has been reported to give a good initial therapeutic response.

The first object of the present investigation was to determine whether these drugs, which are virtually inactive *in vitro*, owe their therapeutic activity to their capacity to become active in an intracellular environment. For, it is well recognized that many of the tubercle bacilli present in lesions are situated intracellularly, notably in the monocytes.

Interest in pyrazinamide has quickened following the reports of McDermott and his associates (9) and of Schwartz and Moyer (10) that pyrazinamide, given in combination with isoniazid, produces a therapeutic response in man better than that obtained with any other known drug combination. A possible explanation is provided by the investigations of McCune and his associates (11*a*, *b*), who found that treatment with pyrazinamide-isoniazid produces an apparent sterilization of the tissues of tuberculous mice. No other drug or drug combination appears capable of this effect.

The second part of this report provides additional evidence, obtained by an *in vitro* method, that pyrazinamide and isoniazid behave synergistically in their bactericidal effect upon intracellular tubercle bacilli.

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MATERIALS AND METHOD

Titration of Intracellular Growth Inhibition in Fluid Monocyte Cultures

In the experiments seeking to demonstrate inhibitory activity of pyrazinamide or nicotinamide against intracellular tubercle bacilli, fluid cultures of rabbit monocytes, which had ingested living tubercle bacilli, were maintained in center-well culture chambers in nutrient medium containing various concentrations of one or other of the two drugs. The methods used were basically those of previous studies (12–14).

Preparation of cell suspensions: As the two drugs, at the concentrations used in these experiments, are devoid of inhibitory activity against extracellular tubercle bacilli, care was needed to reduce the extracellular organisms to a minimum in preparing a population of parasitized monocytes. The latter were obtained from oil-induced peritoneal exudates in normal adult rabbits. Known numbers of monocytes, suspended in fresh rabbit serum, were mixed with an equal number of highly dispersed tubercle bacilli, strain H37Rv. The bacilli were prepared from seven- to ten-day Tween®-albumin cultures by removing the aggregated bacilli in three or four stages by decanting the supernatant after light centrifuging and subjecting it to further centrifugation. The mixture of cells and bacilli was rotated in a paraffin-lined tube at 37°C. for twenty to thirty minutes to allow phagocytosis to occur. A large volume of chilled Hanks' solution (15) was then added to the centrifuge tube and, after mixing, the cell suspension was deposited by centrifuging for three minutes (200 g.). The deposited cells were washed three times in this way to remove as many as possible of the uningested bacilli. The parasitized cell suspension was diluted to contain 1.5×10^6 monocytes per ml. and was then distributed in center-well culture chambers (12).

Preparation of medium: Medium, consisting of 50 per cent heat-inactivated rabbit serum in Hanks' solution (15) and containing test concentrations of either pyrazinamide or nicotinamide,[2] was then added to the chambers. The cultures were incubated at 37.5°C. The medium was changed after twelve hours, and then at twenty-four or forty-eight hour intervals for the duration of the experiment.

pH of medium: The pH of the culture medium was adjusted initially to 7.4 by gassing with a mixture of oxygen containing 5 per cent carbon dioxide. During incubation, the pH was not allowed to fall below pH 7.0.

Bacterial counts: Within the first twenty-four hours, before the onset of bacterial multiplication (16), one or more cultures were fixed and stained with carbolfuchsin to show the initial number of bacilli present in the monocyte population. After further incubation, random cultures were fixed and stained in the same way. Bacterial counts were made on the cultures by counting the bacilli present in individual monocytes in a random sample of approximately 500. From the data obtained, the percentage of infected monocytes, the average number of bacilli per monocyte, and the coefficient of increase in bacterial numbers were calculated for control cultures and for cultures treated with varying concentrations of drug. The coefficient of increase expresses the increase in bacterial numbers during incubation; for a given period it is the average number of bacilli found per monocyte divided by the average number of bacilli per monocyte present before the onset of growth. Its significance has been discussed previously (14).

The Effect of Pyrazinamide and Isoniazid on the Intracellular Survival Rate of Tubercle Bacilli in Agar Cultures of Monocytes

To test for a synergistic effect between pyrazinamide and isoniazid, the method of survival counts used in an earlier study (13) could not be applied here in an unmodified form.

[2] Supplies of pyrazinamide were generously provided by Dr. Walsh McDermott, Cornell University Medical College, New York.

In the previous method (13) heavily infected monocytes were maintained on glass in a fluid medium in center-well culture chambers. After a period of exposure to one or both drugs, the medium was removed and the monocytes were overlaid by a film of oleic-acid–albumin-agar medium. Upon further incubation, monocyte residues which contained viable bacilli gave rise to colonies of tubercle bacilli. Counts on these cultures gave a measure of the survival rate of intracellular bacilli.

In cultures of this sort heavily parasitized monocytes inevitably occur. As pyrazinamide is unable to inhibit growth of such dense aggregates of intracellular bacilli, extracellular organisms appear in the cultures; these also are uninhibited by pyrazinamide. It is necessary, therefore, to use very lightly parasitized monocytes. This in itself introduces a further problem in that isoniazid is so powerfully bactericidal for intracellular bacilli that a large number must be sampled in order to detect an added bactericidal effect when pyrazinamide is added to isoniazid in the culture medium. These limitations of technique were obviated in part by the method adopted.

Preparation of agar cultures of parasitized monocytes: Lightly parasitized monocytes were maintained in flasks on a solid agar medium containing test concentrations of drug. Samples were taken, examined for motility of the monocytes, and transferred to slide cells at intervals. They were then overlaid by oleic-acid–albumin agar and incubated until colonies of bacilli appeared.

Four lots of media were prepared, each consisting of 50 per cent heat-inactivated rabbit serum in Hanks' solution (15), solidified with 1.0 per cent Difco® agar (solvent extracted), and containing 1.0 γ of streptomycin per ml. The four media contained either no added test drug, 25 γ of pyrazinamide per ml., 10 γ of isoniazid per ml., or 25 γ of pyrazinamide plus 10 γ of isoniazid per ml. The agar was dissolved by steaming in Hanks' balanced salt solution without added bicarbonate. Appropriate concentrations of pyrazinamide and isoniazid were then added, and the four solutions were sterilized by autoclaving at 15 pounds' pressure for fifteen minutes. Sodium bicarbonate and streptomycin were added to heat-inactivated rabbit serum (56°C. for thirty minutes) to a concentration of 0.035 per cent and 2.0 γ per ml., respectively. The serum-bicarbonate-streptomycin complex was sterilized by Seitz filtration. Four volumes of this mixture were transferred to sterile flasks and an equal volume of one of the four agar-salt-drug complexes was added to each at 50°C. The solutions were mixed and distributed in duplicate in 15 ml. volumes in 50 ml. sterile Erlenmeyer flasks and were allowed to solidify on a level surface. The resultant medium had a final pH of 7.4, was moist but firm, and contained a background concentration of streptomycin of 1.0 γ per ml. The streptomycin was introduced to restrain unwanted multiplication of extracellular tubercle bacilli during the course of the experiment. At this concentration streptomycin is without effect upon intracellular bacilli (12, 17).

A suspension of rabbit monocytes was lightly parasitized with tubercle bacilli, strain H37Rv, in the manner described. A volume of 0.5 ml. containing 3×10^4 monocytes was placed on the surface of each flask of medium and gently tilted to distribute it evenly over the entire surface of the agar. The flasks were stoppered with sterile rubber bungs and left undisturbed on a level surface at 37.5°C.

Transplantation of monocytes to oleic-acid–albumin agar medium: After thirty hours' incubation and again after seventy-two hours' incubation the flasks were gently tilted to float the cells from the surface, and a 0.2 ml. sample of the cell suspension was removed from each flask to a centrifuge tube containing 25 ml. of sterile Hanks' solution. After mixing, the tubes were centrifuged; the supernatant was then decanted, and the deposited cells were resuspended in another 25 ml. volume of Hanks' solution. Washed in this way the cell suspensions were freed from all but traces of drug. After a further centrifuging, the deposited cells were resuspended in the small volume of Hanks' solution which remained after decanting. A sample of this suspension was examined in a slide-coverslip preparation at 37°C. to ensure that the monocytes had survived in a healthy state during the period of incubation. Except in one case in which bacterial contamination had occurred, the monocytes in all cultures were healthy and motile at the end of the period of incubation. A drop of the

cell suspension containing 1–2 × 10^6 monocytes was then transferred to each of two slide cells (center-well chambers without a central ring (12)) and was spread in a thin film over the entire surface of the bottom coverslip. The film was allowed to dry momentarily and was then covered by a thin layer of oleic-acid–albumin agar medium cooled to 45°C. The top coverslip was introduced, the chambers were sealed completely with paraffin wax, and the cultures were incubated at 37.5°C.

Viable counts: After incubation for twenty-two days, each chamber was filled to capacity with 10 per cent formalin-saline and examined with a phase-contrast microscope. Monocyte residues from which colonies of tubercle bacilli had grown were immediately recognizable (13). In a random sample of at least 1,000 monocyte residues, the number yielding characteristic bacterial colonies, and therefore the number containing viable tubercle bacilli, was expressed as a percentage of the total. This percentage was averaged for the duplicate samples taken from each of the duplicate flask cultures of monocytes. In this way each test concentration of drug was tested in quadruplicate.

RESULTS

The samples of pyrazinamide and nicotinamide used in these experiments were tested for inhibitory activity against strain H37Rv under conventional conditions in Tween-albumin medium. Neither drug showed any inhibition of growth after ten days' incubation in concentrations as high as 500 γ per ml.

Growth Inhibition of Intracellular Tubercle Bacilli by Nicotinamide and Pyrazinamide

Nicotinamide: The inhibitory activity of nicotinamide against intracellular tubercle bacilli was titrated three times. On each occasion the same end-point was obtained. The results of one experiment are recorded in table 1.

A coefficient of increase of 1.2, as obtained in the presence of 12.5 γ of nicotinamide per ml., represents virtually complete inhibition of bacterial growth. A small redistribution of bacilli inevitably occurs during the first forty-eight hours by the death of granulocytes present in small numbers in the original cell population. Re-phagocytosis of bacilli liberated in this way would account for most of the apparent increase in bacterial numbers observed at this concentration of drug.

TABLE 1

NUMBER OF INTRACELLULAR BACILLI PRESENT IN MONOCYTE CULTURES INCUBATED FOR SIX DAYS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF NICOTINAMIDE

	Period of Incubation					
	6 hours	6 days				
		Concentration of Nicotinamide (γ/ml.)				
		50	25	12.5	6.25	0
Average number of bacilli per monocyte	0.89	0.6	0.6	1.0	1.5	2.3
Percentage of monocytes infected	27	24.0	22.0	26.0	28.0	35.0
Coefficient of increase	—	0.8	0.8	1.2	1.9	3.0

After six days' incubation all remaining cultures in this experiment were washed free from drug and incubated for a further five days in medium free from drug. The bacterial counts obtained on the eleventh day give some indication of the degree of damage done to the intracellular bacilli during the period of exposure to nicotinamide (table 2). It will be seen that at drug concentrations higher than 12.5 γ per ml. the size of the bacterial population remained almost unchanged. In the stained culture, microscopic examination showed that, when multiplication had occurred following withdrawal of the drug, it was confined to an occasional monocyte. In the majority of the monocytes the bacilli were small and coccal in form, a change which is known to occur during the intracellular digestion of dead tubercle bacilli (12). It is apparent, therefore, that almost all of the bacterial population had been killed during a six-day exposure to nicotinamide at a concentration of 25 γ per ml., and that drug concentrations of 12.5 and 6.25 γ per ml. had interfered markedly with bacillary growth.

Pyrazinamide: The inhibitory activity of pyrazinamide against intracellular tubercle bacilli was also titrated in three experiments and in several preliminary experiments. In each case an identical end-point was obtained. In the experiment reported it was intended to make viable counts of the intracellular bacterial population after exposure to pyrazinamide and isoniazid, both singly and in combination. For reasons already given, this approach to testing for synergism between these two drugs was unsuccessful. Although survival counts were not obtained in this experiment, the bacterial counts after exposure to isoniazid and pyrazinamide plus isoniazid are informative and have been included in table 3.

The apparent small increase in bacterial numbers which was found at pyrazinamide concentrations of 12.5 and 25 γ per ml. is probably again the result of redistribution of bacilli. For, when multiplication is found, it is attended by morphologic changes in the bacilli themselves (12, 16); here no such evidence of bacterial multiplication was found. It will be noted also that in these cultures there was no increase in the percentage of monocytes containing bacilli; this is a crude indication that bacterial growth had not occurred.

TABLE 2

NUMBER OF INTRACELLULAR BACILLI PRESENT IN MONOCYTE CULTURES INCUBATED FOR SIX DAYS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF NICOTINAMIDE AND FOR A FURTHER FIVE DAYS IN THE ABSENCE OF DRUG

	Period of Incubation					
	6 hours	11 days				
		Concentration of Nicotinamide (γ/ml.)				
		50	25	12.5	6.25	0
Average number of bacilli per monocyte	0.89	0.7	0.7	2.0	2.0	>9.1
Percentage of monocytes infected	27	25.0	24.0	30.0	36.0	63.0
Coefficient of increase	—	0.9	0.9	2.5	2.6	>11.7

TABLE 3

NUMBER OF INTRACELLULAR BACILLI PRESENT IN MONOCYTE CULTURES INCUBATED FOUR DAYS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF PYRAZINAMIDE, ISONIAZID, AND PYRAZINAMIDE PLUS ISONIAZID

	Period of Incubation							
	24 hours	4 days						
		Drug Concentration (γ/ml.)						
		Pyrazinamide					Isoniazid	Pyrazinamide plus Isoniazid
		50	25	12.5	6.25	0	10	12.5/10
Average number of bacilli per monocyte	1.1	0.9	1.2	1.2	2.9	4.1	0.6	0.5
Percentage of monocytes infected	37.0	37.0	37.0	36.0	50.0	55.0	27.0	25.0
Coefficient of increase	—	0.8	1.1	1.1	2.6	3.7	0.6	0.5

In the presence of 50 γ of pyrazinamide per ml., morphologic changes in the bacilli suggested that some digestion of bacilli had occurred. This was confirmed in the bacterial counts by a coefficient of increase of less than one. In cultures containing isoniazid, digestion of bacilli was even more marked. In the presence of isoniazid alone, almost half the contained bacilli had disappeared from the monocytes while, in the cultures containing pyrazinamide in addition to the isoniazid, slightly more than half the bacilli had been digested. This small difference between the coefficients of increase for cultures containing isoniazid and pyrazinamide-isoniazid cannot be taken as an indication of a potentiation of effect due to the action of two drugs. Even dead bacilli are known to persist for long periods within living monocytes. To detect a synergistic effect between two drugs, it is necessary to determine the survival rate of the bacterial population.

Survival Rate of Intracellular Tubercle Bacilli Exposed to Pyrazinamide and Isoniazid

The percentages of monocytes found to contain viable tubercle bacilli after cultivation on agar medium containing pyrazinamide (25 γ per ml.), isoniazid (10 γ per ml.), or both drugs together at the same concentrations are set out in table 4, together with the percentages found in untreated control cultures. Counts for the thirty-hour and seventy-two-hour exposure periods appear in separate columns. The results are shown graphically in figure 1.

After thirty hours' exposure, neither pyrazinamide nor isoniazid caused a significant fall in the percentage of monocytes containing viable bacilli. A similar lag in the bactericidal effect of isoniazid has previously been reported (13, 18). Simultaneous exposure to both drugs, however, caused a large fall in viable count. In the monocyte sample taken from one agar culture containing isoniazid

TABLE 4

PERCENTAGE OF MONOCYTE RESIDUES YIELDING COLONIES OF TUBERCLE BACILLI AFTER THIRTY AND SEVENTY-TWO HOURS IN UNTREATED CULTURES AND CULTURES EXPOSED TO PYRAZINAMIDE (25 γ PER ML.), ISONIAZID (10 γ PER ML.), AND THE TWO DRUGS COMBINED AT THE SAME CONCENTRATION

	Percentage of Monocyte Residues Yielding Bacterial Colonies	
	Period of Exposure	
	30 hours	72 hours
Control	12.5	10.0
	Contaminated	11.0
Pyrazinamide	9.1	2.0
	10.7	3.5
Isoniazid	9.9	0.5
	10.6	0.3
Pyrazinamide/isoniazid	1.9	0
	0	0

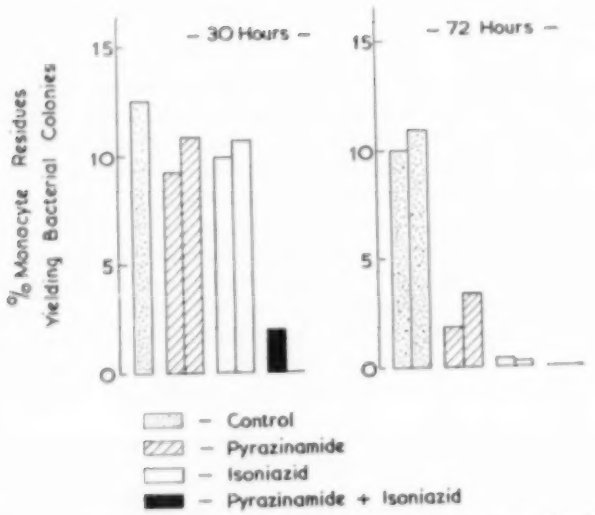

FIG. 1. The percentage of monocytic residues from which colonies of tubercle bacilli grew after transplantation to oleic-acid–albumin–agar medium. The parasitized monocytes had previously been incubated for thirty or seventy-two hours on an agar medium free from test drugs (control) or containing pyrazinamide (25 γ per ml.), isoniazid (10 γ per ml.), or pyrazinamide (25 γ per ml.) plus isoniazid (10 γ per ml.).

and pyrazinamide, no bacterial colonies developed in the entire population of approximately 2×10^5 monocyte residues; in the other, the viable count had fallen by more than 80 per cent.

The counts obtained after seventy-two hours' exposure to drugs are less con-

vincing since the effect of each drug acting alone had produced a marked fall in viable count. However, the cultures exposed simultaneously to both drugs yielded no colonies in a total population of almost half a million monocyte residues.

DISCUSSION

In seeking to explain the discrepancy between the antituberculous activity of pyrazinamide *in vivo* and its apparent absence *in vitro*, McDermott and Tompsett (19) showed that the inhibitory activity of pyrazinamide *in vitro* was greatly enhanced when the pH of the culture medium was reduced to relatively low levels. From the results of the present experiments the inhibitory activity of pyrazinamide and nicotinamide against intracellular tubercle bacilli appears to be comparable with that found by McDermott and Tompsett for extracellular bacilli grown in Tween-albumin medium at pH 5.5. Whether the activation of pyrazinamide intracellularly is determined by H-ion concentration or is due to some other mechanism remains unknown. Throughout the period of observation, the pH of the culture medium was never allowed to fall below 7.0. The culture media contained phenol red so that any change in pH was immediately recognizable. In cell populations of the size used in these experiments, the pH does not fall below 7.0 before the third day of incubation; and, since medium was changed at twenty-four- or forty-eight-hour intervals, the pH in the ambient culture medium was presumably not a factor in determining the activity of the drugs against organisms segregated within monocytes.

It has been claimed that very high H-ion concentrations may occur within phagocytic cells *in vivo* (20). However, Smith, Willis, and Lewis (21) found in supravital preparations of chicken macrophages that intracellular tubercle bacilli of avian type gave a color reaction to neutral red which corresponded to a pH near neutrality. Attempts were made in this laboratory to reproduce this finding, but no staining of intracellular tubercle bacilli with neutral red or with any of a variety of indicator dyes could be obtained; nor was any indication given that the pH in the vicinity of the tubercle bacillus was different from that prevailing in the cytoplasm elsewhere. On the other hand, Sprick (22) has recently shown that the reaction within monocytes in the vicinity of ingested tubercle bacilli may be as low as pH 4.7. In such circumstances H-ion concentration may be the factor responsible for intracellular activation of pyrazinamide just as McDermott and Tompsett have shown for extracellular organisms *in vitro*.

Reports from other laboratories indicate that pyrazinamide is somewhat less effective in controlling tuberculosis in the guinea pig than in the mouse. If there is a true difference of response in these two species, it may be due to differences in the nature of the tuberculous lesion in them. In the mouse, necrosis is seen only when animals are infected with excessively large inocula. Apart from this circumstance, the tubercle bacillus is almost exclusively an intracellular parasite in this host species. On the other hand, necrosis is an early and extensive feature of the lesions in guinea pigs. As pyrazinamide may be active only against intracellular bacilli *in vivo*, a difference in the response of these animal species is understandable.

McCune and his associates (11*a*, *b*) found that simultaneous treatment with pyrazinamide and isoniazid produced apparent sterility of the tissues of tuberculous mice, an effect not obtainable with either drug alone or with any drug combination that did not include pyrazinamide. The survival counts reported here for intracellular bacilli support these findings. Ideally the drugs should have been tested at various concentrations. The concentrations employed, however, have a rational basis in that the bactericidal action of isoniazid is, within limits, independent of concentration (13). Hence, no advantage would accrue from testing at various drug concentrations. It was decided, therefore, to test at the concentration commonly achieved in the body fluids during therapy. For pyrazinamide preliminary tests had shown very little immediate bactericidal effect; so that here, too, there was no obvious advantage in varying the drug concentration. Instead, a mildly lethal but fully bacteriostatic concentration was chosen for test in combination with isoniazid. The size of the bacterial populations studied was relatively small so that it cannot be definitely concluded that pyrazinamide and isoniazid are synergistic in their bactericidal effect upon intracellular tubercle bacilli. In each of five attempts to find a satisfactory method of examining this question there was, however, evidence of an accelerated death rate in those cultures which contained both pyrazinamide and isoniazid.

The generation time of virulent tubercle bacilli in monocytes is approximately twenty-four hours in cultures of the type used in these experiments. It is presumed, therefore, that conditions for growth in this test system are almost optimal. Since a bactericidal effect is obtained with isoniazid only in actively growing cultures (13, 18), it is apparent that the results obtained under the present experimental conditions may not be a quantitative reflection of the effects produced with these drugs *in vivo*, when growth conditions may be far from optimal. The results do indicate, however, that pyrazinamide and nicotinamide are potentially capable of controlling the growth of intracellular bacilli *in vivo* and that, under suitable conditions, pyrazinamide may potentiate the antibacterial effect of isoniazid.

SUMMARY

Pyrazinamide and nicotinamide, which are almost devoid of tuberculostatic activity against tubercle bacilli grown under ordinary cultural conditions, were found to possess a high level of activity against tubercle bacilli, strain H37Rv, grown in isolated rabbit monocytes *in vitro*. Compared with isoniazid, pyrazinamide was found to have only a slow bactericidal effect upon intracellular bacilli under these conditions. Combined with isoniazid, pyrazinamide resulted in an apparent increase in the bactericidal effect of isoniazid.

SUMARIO

La Activación Intracelular de la Piracinamida y la Nicotinamida

La piracinamida y la nicotinamida, que están casi desprovistas de actividad tuberculostática contra los bacilos tuberculosos cultivados en condiciones ordinarias, mostraron poseer un alto índice de actividad contra bacilos tuberculosos, cepa H37Rv, cultivados en monocitos de conejo, aislados, *in vitro*. Comparada con la isoniacida, la piracinamida no

mostró más que efecto bactericida lento sobre los bacilos intracelulares en esas condiciones. Combinada con la isoniacida, la piracinamida logró un aumento aparente del efecto bactericida de la isoniacida.

RESUME

L'activation intracellulaire de la pyrazinamide et de la nicotinamide

Il a été observé que la pyrazinamide et la nicotinamide, qui sont presque dépourvues d'activité tuberculostatique contre les bacilles tuberculeux cultivés dans les conditions usuelles, possédaient un haut degré d'activité contre les bacilles tuberculeux, souche H37Rv, cultivés *in vitro* dans les monocytes isolés du lapin. Dans ces conditions, et par comparaison avec l'isoniazide, il a été observé que la pyrazinamide n'avait sur les bacilles intracellulaires qu'une action bactéricide ralentie. Associée à l'isoniazide la pyrazinamide a provoqué une augmentation apparente de l'effet bactéricide de l'isoniazide.

Acknowledgments

These experiments were performed with the assistance of Mr. A. A. Argyle upon a suggestion made by Dr. Walsh McDermott. I am indebted to Dr. L. F. Dodson for advice in the preparation of this paper.

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MEASUREMENT OF FUNCTIONAL RESIDUAL CAPACITY[1]

A Comparison of Two Closed-Circuit Helium-Dilution Methods

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INTRODUCTION

The determination of the functional residual capacity (FRC) has been of increasing interest to the internist, chest physician, and thoracic surgeon, for it gives one a numerical value for the gas retained within the lungs at the end of the usual quiet expiration.

It is known that an irreversible increase in functional residual capacity occurs with emphysema, and the magnitude of this measurement has been made the basis of grading the severity of the disease (1). In bronchial asthma and in certain other conditions a rise in the functional residual capacity may be reversible.

Simple clinically applicable methods for the determination of functional residual capacity are of importance, for the finding of an excessive volume of gas retained within the lungs may indicate a serious disturbance of the mechanics of breathing and intrapulmonary gas distribution.

The functional residual capacity has been measured by closed-circuit technique for many years. The method of Van Slyke and Binger, which Christie (2) considered the most reliable, made use of hydrogen as the indicator gas. This was modified by Christie with nitrogen as the indicator gas, eliminating the risk of explosion. Herrald and McMichael (3) developed a constant-volume modification, using hydrogen and a katharometer for analysis. This constant-volume modification eliminated the error due to the "oxygen storage effect" commented upon by Lassen and associates (4). Gilson and Hugh-Jones (5) reported a similar method, using helium and a katharometer for analysis with oxygen as the diluent in a closed-circuit system. Meneely and Kaltreider (6) employed a similar method but used air instead of oxygen as the diluent.

This study is a comparison of the method of Meneely and Kaltreider, using air in the spirometer circuit (Method *A*), with a modification of the method of Gilson and Hugh-Jones, using oxygen in the circuit (Method *O*). The study was made to determine whether the simpler air dilution method was satisfactory for clinical investigation.

PLAN OF INVESTIGATION

Method

The principle of both methods for determination of the functional residual capacity is the same and well known. A measured amount of helium is introduced into the spirometer and the dead space of the apparatus (V_D) is determined by observing the dilution of helium.

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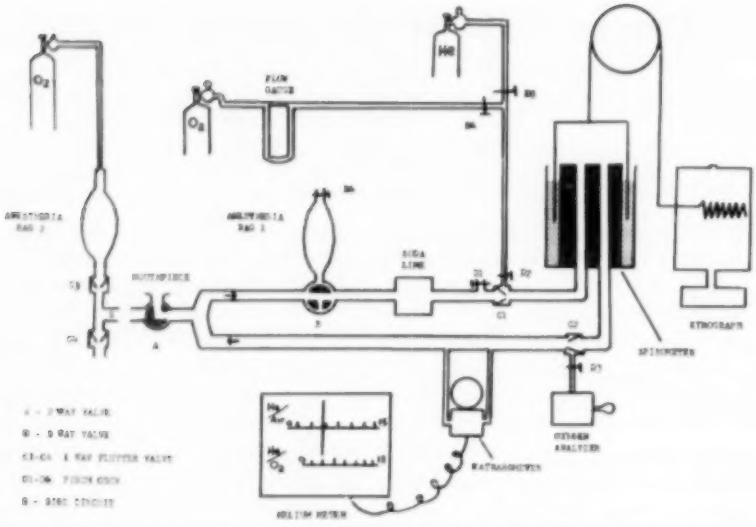

FIG. 1. Diagram of apparatus used for determination of functional residual capacity.

The patient then breathes into the circuit and the functional residual capacity can be calculated from the further dilution of the helium after it is distributed throughout the lung-spirometer system.

Apparatus

The apparatus used (figure 1) is that of Meneely and Kaltreider (6), modified so that determinations with helium in oxygen can also be performed.

The main circuit is fitted with low-resistance valves.[3] A nine-liter respirometer[2] with reduced internal dead space is used. The katharometer[4] contains wet air as a standard. The galvanometer[3] has a double scale, one to record per cent helium measured in air, the other reads per cent helium in oxygen. In either case, the zero point on the helium meter is adjusted with the circuit filled with air saturated with water vapor. The gas mixtures are distributed throughout the circuit, including the katharometer, by means of a mixing bag. The soda lime cannister removes carbon dioxide, which would affect the response of the katharometer. The oxygen analyzer[4] is used only for oxygen readings in Method *O*. A simple flow gauge is used to match the oxygen inflow to the patient's oxygen consumption during each determination.

Preliminary mixing is accomplished by shifting the gas back and forth between the spirometer and a previously evacuated anesthesia bag 1. Finally the gas in the anesthesia bag is returned to the spirometer. Valve *A* is used to turn the patient into the system.

The spirometer circuit is filled with oxygen for Method *O* and side circuit *S* is connected to the mouthpiece valve *A* so that the patient can breathe oxygen.

To keep the dead space of the apparatus constant, the water level in the spirometer and the weight of soda lime of standard pellet size are kept constant.

[3] Manufactured by Warren E. Collins Co., Boston, Massachusetts.
[2] Manufactured by Cambridge Instrument Co., New York, New York.
[4] Manufactured by Beckman Instrument Co., Pasadena, California.

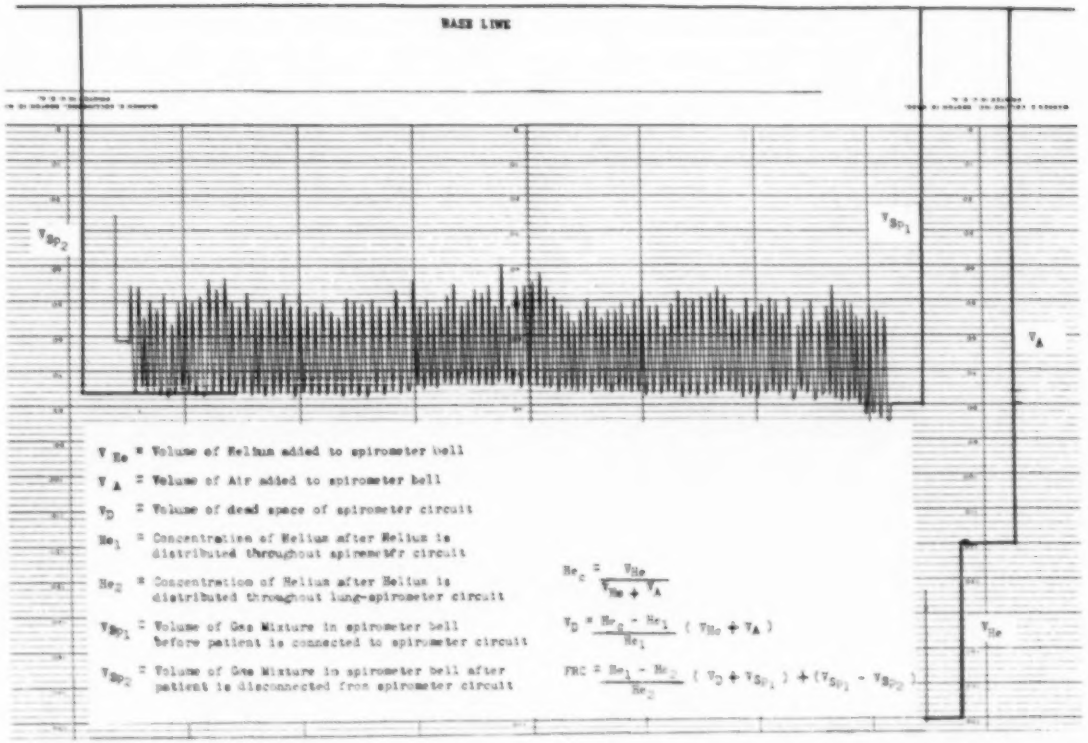
BASE LINE
V_{Sp_2}
V_{Sp_1}
V_A
V_{He}
V_{He} = Volume of Helium added to spirometer bell
V_A = Volume of Air added to spirometer bell
V_D = Volume of dead space of spirometer circuit
He_1 = Concentration of Helium after Helium is distributed throughout spirometer circuit
He_2 = Concentration of Helium after Helium is distributed throughout lung-spirometer circuit
V_{Sp_1} = Volume of Gas Mixture in spirometer bell before patient is connected to spirometer circuit
V_{Sp_2} = Volume of Gas Mixture in spirometer bell after patient is disconnected from spirometer circuit
$He_c = \frac{V_{He}}{V_{He} + V_A}$
$V_D = \frac{He_c - He_1}{He_1} (V_{He} + V_A)$
$FRC = \frac{He_1 - He_2}{He_2} (V_D + V_{Sp_1}) + (V_{Sp_1} - V_{Sp_2})$

FIG. 2. Respiratory tracing employing Method A. Formulas for determining functional residual capacity

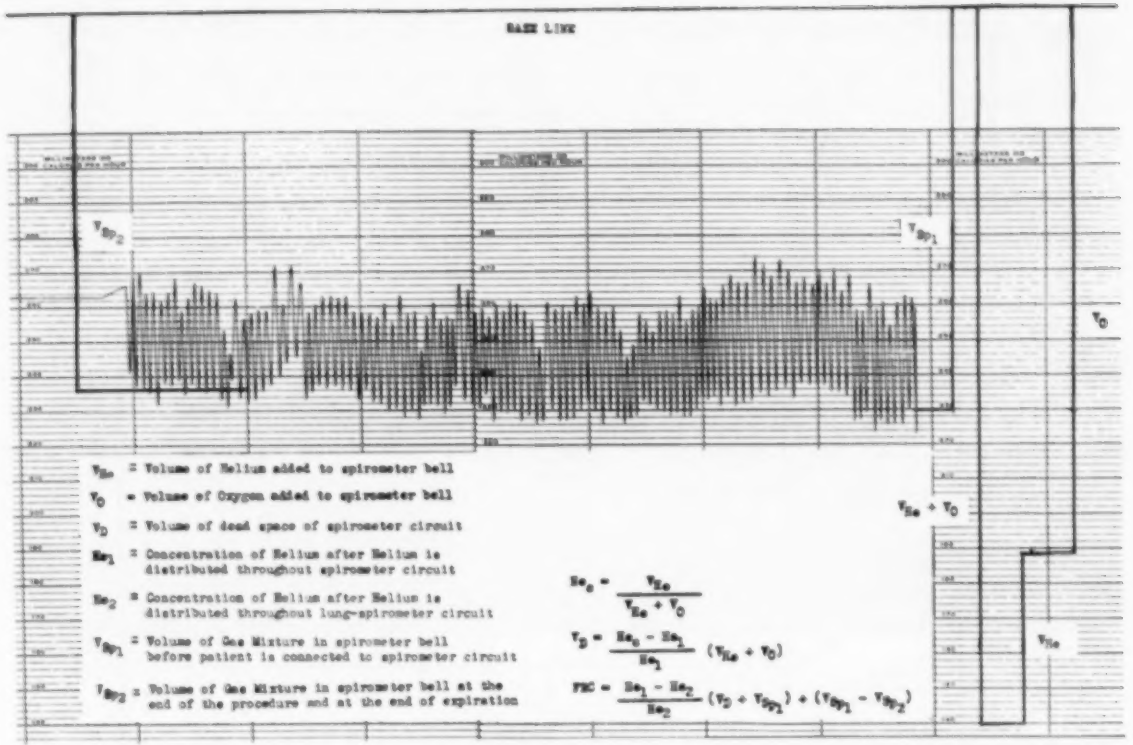
BASE LINE
V_{Sp_2}
V_{Sp_1}
V_0
$V_{He} + V_0$
V_{He}
V_{He} = Volume of Helium added to spirometer bell
V_0 = Volume of Oxygen added to spirometer bell
V_D = Volume of dead space of spirometer circuit
He_1 = Concentration of Helium after Helium is distributed throughout spirometer circuit
He_2 = Concentration of Helium after Helium is distributed throughout lung-spirometer circuit
V_{Sp_1} = Volume of Gas Mixture in spirometer bell before patient is connected to spirometer circuit
V_{Sp_2} = Volume of Gas Mixture in spirometer bell at the end of the procedure and at the end of expiration
$He_0 = \frac{V_{He}}{V_{He} + V_0}$
$V_D = \frac{He_0 - He_1}{He_1} (V_{He} + V_0)$
$FRC = \frac{He_1 - He_2}{He_2} (V_D + V_{Sp_1}) + (V_{Sp_1} - V_{Sp_2})$

FIG. 3. Respiratory tracing employing Method *O*. Formulas for determining functional residual capacity

Procedure

Method A: The spirometer circuit is flushed with air and the helium indicator is adjusted to zero on the "helium in air" scale. The volume of helium (V_{He}) and air (V_A) introduced are measured. The gases are mixed, and the concentration of helium (He_1) is recorded. The volume of each gas can be read easily from the excursion of the pen on the kymograph paper. The final concentration of the helium mixed with the dead space gas must be within the range of the katharometer (less than 15 per cent). The spirometer should contain approximately 1,000 cc. of gas. A base line is recorded on the kymograph paper. The patient who has been resting in a supine position is turned into the system and enough oxygen is added to maintain the respiratory tracing horizontal, and thus a constant volume system is maintained. Re-breathing continues for seven minutes, or until the percentage of helium in air remains constant for three minutes. The patient is then removed from the system, the gases are mixed, and the concentration of helium (He_2) is determined. The dead space of the apparatus (V_D) and the functional residual capacity, uncorrected for dead space of the mouthpiece, temperature, or water vapor pressure, are then determined by use of simple dilution formulas (figure 2).

Method O: The spirometer is again flushed with air, and the helium meter adjusted to zero on the "helium in air" scale. A switch on the helium meter is then turned to the "helium in oxygen" scale. The spirometer is then flushed with oxygen until the oxygen analyzer records a partial pressure of oxygen approximating barometric pressure. The indicator then records 0 per cent helium on the "helium in oxygen" scale. A base line is recorded, then helium and oxygen are added and measured. After mixing, the concentration of helium (He_1) is recorded, and from these data the dead space is determined.

The patient who has been breathing pure oxygen through the side circuit for seven minutes is then turned into the spirometer and the procedure is continued as in Method *A*. At the end the per cent of helium (He_2) and the per cent of oxygen are determined. The per cent of oxygen plus the per cent of helium subtracted from 100 is the per cent of nitrogen in the final mixture. Since for every 10 per cent increase in nitrogen in the final mixture the helium indicator reads 0.14 per cent too low, an appropriate correction must be made. The functional residual capacity is then computed by dilution formulas (figure 3).

RESULTS

Thirty-six determinations of the dead space were made by both methods for the measurement of functional residual capacity (table 1).

Duplicate determinations of functional residual capacity were carried out by each of the two methods on 16 patients, 14 of whom had no disease that would interfere with pulmonary mixing and 2 of whom had pulmonary emphysema. All determinations were done consecutively, first by Method *A* and then by Method *O*, over a two-hour period.

The functional residual capacity for each method was calculated, using the mean V_D determined by the same method. The results appear in table 2. The FRC_A and FRC_O were computed, using the mean of V_{D_A} and V_{D_O}, i.e., $\frac{V_{D_A} + V_{D_O}}{2}$, since the dead space of the apparatus was actually constant throughout, despite the fact that mean dead space as measured by the two methods differed slightly. The results appear in table 3.

Dead space determined by both methods should have been identical. The difference between the mean of V_{D_A} and V_{D_O} was 74 cc. with a standard error of the difference of the means of plus 19 cc., indicating a statistically significant difference. The cause of this is not definitely known, but it may have been due

TABLE 1

RESULTS OF 36 DETERMINATIONS OF DEAD SPACE BY METHOD *A* AND BY METHOD *O*

	Method *A*	Method *O*
	cc.	cc.
Mean V_D	3,354	3,280
Standard deviation	±90	±68
Error of the mean	±15	±11

Difference of the means $V_{D_A} - V_{D_O} = 74$ cc.
Standard error of the difference of the means = ±19 cc.

TABLE 2

COMPARISON OF FUNCTIONAL RESIDUAL CAPACITY RESULTS BY METHODS *A* AND *O*, USING THE MEAN DEAD SPACE DETERMINED BY METHODS *A* AND *O*

Patient Number	FRC_A (V_D = 3,354 cc.)	Difference of Pair	Mean	Error of Pair $\frac{½ \text{ Difference}}{\text{Mean}} \times 100\%$	FRC_O (V_D = 3,280 cc.)	Difference of Pair	Mean	Error of Pair $\frac{½ \text{ Difference}}{\text{Mean}} \times 100\%$	Difference Between Means $FRC_A - FRC_O$
		cc.	cc.			cc.	cc.		
1	2,416	403	2,215	9.1	2,160	37	2,142	0.9	+73
	2,013				2,123				
2	3,736	283	3,595	3.9	3,357	99	3,308	1.5	+287
	3,453				3,258				
3	3,181	148	3,255	2.3	3,066	83	3,025	1.4	+230
	3,329				2,983				
4	1,138	75	1,101	3.5	911	37	930	2.0	+171
	1,063				948				
5	1,867	113	1,811	3.2	1,696	34	1,713	0.9	+98
	1,754				1,730				
6	2,509	87	2,466	1.8	2,356	216	2,464	4.4	+2
	2,422				2,572				
7	2,921	147	2,848	2.6	2,673	13	2,680	0.3	+168
	2,774				2,686				
8	2,758	207	2,655	3.9	2,801	136	2,733	2.5	−78
	2,551				2,665				
9	3,312	43	3,291	0.7	3,300	70	3,265	1.1	+26
	3,269				3,230				
10	1,708	82	1,667	2.5	1,693	82	1,652	2.5	+15
	1,626				1,611				
11	1,879	33	1,896	0.9	1,715	25	1,728	0.8	+168
	1,912				1,740				
12	3,369	117	3,311	1.8	3,150	17	3,159	0.3	+152
	3,252				3,167				
13	2,564	58	2,593	1.1	2,559	30	2,574	5.8	+19
	2,622				2,589				
14	2,531	106	2,584	2.1	2,399	71	2,435	1.5	+149
	2,637				2,470				
15	2,086	21	2,097	0.5	2,146	1	2,146	0.02	−49
	2,107				2,145				
16	2,928	9	2,933	0.2	3,084	283	2,943	4.8	−10
	2,937				2,801				

TABLE 3

COMPARISON OF FUNCTIONAL RESIDUAL CAPACITY RESULTS BY METHODS *A* AND *O*, USING THE AVERAGE OF THE MEAN DEAD SPACE DETERMINED BY METHODS *A* AND *O*

Patient Number	FRC_A (V_D = 3,317 cc.)	Difference of Pair	Mean	Error of Pair $\frac{\frac{1}{2}\text{ Difference}}{\text{Mean}} \times 100\%$	FRC_O (V_D = 3,317 cc.)	Difference of Pair	Mean	Error of Pair $\frac{\frac{1}{2}\text{ Difference}}{\text{Mean}} \times 100\%$	Difference Between Means $FRC_A - FRC_O$
		cc.	cc.			cc.	cc.		
1	2,398	398	2,199	9.0	2,172	34	2,155	0.8	+44
	2,000				2,138				
2	3,706	275	3,569	3.9	3,377	100	3,327	1.5	+242
	3,431				3,277				
3	3,167	143	3,239	2.2	3,089	87	3,046	1.4	+193
	3,310				3,002				
4	1,130	72	1,094	3.3	917	37	936	2.0	+158
	1,058				954				
5	1,855	114	1,798	3.2	1,708	34	1,725	1.0	+73
	1,741				1,742				
6	2,495	91	2,450	1.9	2,373	214	2,480	4.3	−30
	2,404				2,587				
7	2,907	151	2,832	2.7	2,691	12	2,697	0.2	+135
	2,756				2,703				
8	2,739	203	2,638	3.9	2,819	133	2,753	2.4	−115
	2,536				2,686				
9	3,295	54	3,268	0.8	3,323	66	3,290	1.0	−22
	3,241				3,257				
10	1,695	82	1,654	2.5	1,704	84	1,662	2.5	−8
	1,613				1,620				
11	1,865	34	1,882	0.9	1,726	26	1,739	0.7	+143
	1,899				1,752				
12	3,351	120	3,291	1.8	3,172	20	3,182	0.3	+109
	3,231				3,192				
13	2,545	60	2,575	1.2	2,577	32	2,593	0.6	−18
	2,605				2,609				
14	2,514	109	2,569	2.1	2,412	72	2,448	1.5	+121
	2,623				2,484				
15	2,072	24	2,084	0.5	2,161	1	2,161	0.02	−77
	2,096				2,160				
16	2,986	13	2,913	0.2	3,064	240	2,944	4.2	−31
	2,919				2,824				

to a slight but constant error in adjusting the zero point of the meter for the helium in oxygen determinations. It is believed that the difference between the means of V_{D_A} and V_{D_O} is not significant for clinical studies.

In table 2, V_{D_A} was used in computing FRC_A, and V_{D_O} in computing FRC_O. The difference between the means of duplicate determinations of FRC_A and FRC_O on the same individual is charted in the last column. The largest difference was 287 cc.

If the average of the means of V_{D_A} and V_{D_O}, i.e., $\frac{3,354 + 3,280}{2}$, or 3,317, is

used in computing FRC_A and FRC_O, the largest difference between the means of a pair of duplicate determinations of FRC_A and FRC_O is 242 cc., as noted in table 3.

The error of a pair of determinations of FRC_A and FRC_O is recorded in columns 5 and 9 in tables 2 and 3. This error, a measure of the reliability of the method, is only slightly smaller for Method *O* than for Method *A*.

DISCUSSION

Error due to diminishing volume of the spirometer circuit is eliminated in both methods for the determination of functional residual capacity, since oxygen is added at the same rate as it is consumed. In figures 2 and 3 the maintenance of a horizontal base line in both methods is shown. No correction need be made in He_2 for FRC_A if FRC is less than 3.5 liters, and the correction can be determined quite simply if FRC is larger than 3.5 liters. In Method *A*, there is very little percentage change in composition of the air during a determination, if oxygen is added at the rate at which it is consumed, since there is very little nitrogen excretion by the patient because of the low blood-lung gradient of nitrogen. In Method *O*, in normal subjects, the alveolar nitrogen falls to less than 2 per cent during the period of oxygen breathing (7). Since the rate of excretion of nitrogen after this initial period is approximately 10 cc. per minute, the percentage of nitrogen in the circuit at the end of a determination is generally less than 3 per cent. No correction for He_2 is necessary if the percentage of nitrogen in the circuit is less than 4 per cent at the end of a determination because the correction factor for He_2 in determining functional residual capacity by Method *O* is 0.014 per cent for a 10 per cent increase in nitrogen, and the percentage of helium recorded by the meter can only be read to 0.05 per cent. In patients with emphysema, the percentage of nitrogen in the spirometer at the end of a determination frequently will be greater than 4 per cent and, therefore, He_2 must be corrected, otherwise He_2 will be too low and FRC_O will be too large. Gilson and Hugh-Jones (5) neglected this factor in their analysis of the possible errors in their method of determining functional residual capacity.

The determination of functional residual capacity by Method *O* has a slight advantage because the error due to the respiratory quotient's being less than unity is eliminated (8). With Method *A*, the smaller volume of carbon dioxide given off compared with the volume of oxygen taken up results in a respiratory quotient of less than 1. That factor causes an error of approximately 40 cc. (6).

With Method *O*, the patients must breath through the apparatus for fourteen instead of seven minutes. This increased breathing period makes the determination much more trying for elderly or very ill patients.

In both methods it is necessary to determine the resting expiratory level at the end of a determination by observation of the respiratory tracing. This is frequently difficult in patients with pulmonary disease and may result in an error in functional residual capacity of 100 to 200 cc. This difficulty in estimating

the resting expiratory level may be chiefly responsible for the difference in functional residual capacity obtained by both methods.

Other possible sources of error of the closed circuit method and necessary precautions in the use of the apparatus have been discussed in detail in the reports by Gilson and Hugh-Jones (5) and Meneely and Kaltreider (6) and need no repetition.

The present writers believe that the determination of functional residual capacity by Method *A* is simpler, can be performed more rapidly, is easier for the patient, and is as reliable as Method *O*. Therefore, it appears to be the method of choice for clinical purposes.

SUMMARY

Two modifications of the closed-circuit helium-dilution method for the determination of the functional residual capacity have been compared.

On the basis of the data obtained, it has been concluded that the simpler of the two methods, using helium diluted in room air, is suitable for clinical investigation.

SUMARIO

Medición de la Capacidad Residual Funcional: Comparación de Dos Métodos de Dilución de Helio en Circuito Cerrado

Compáranse dos modificaciones del método de dilución de helio en circuito cerrado para la determinación de la capacidad residual funcional.

A base de los datos obtenidos, se deduce que el más sencillo de los dos métodos, que usa helio diluído en el aire de un aposento, se presta para la investigación clínica.

RESUME

Détermination de la capacité résiduelle fonctionnelle: Comparaison de deux méthodes de dilution de l'hélium en circuit fermé

Deux modifications de la méthode de dilution de l'hélium en circuit fermé pour la détermination de la capacité résiduelle fonctionnelle ont été comparées.

En se fondant sur les données obtenues, les auteurs concluent que la plus simple des deux méthodes, où l'hélium est dilué dans l'air ambiant, convient à l'investigation clinique.

Acknowledgment

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THE PHYSIOLOGIC DISPOSITION OF CYCLOSERINE IN THE HUMAN SUBJECT[1, 2]

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INTRODUCTION

This report deals with a preliminary investigation of the absorption, excretion, and metabolic fate of cycloserine (D-4-amino-3-isoxazolidinone), a drug which has exhibited limited activity in the treatment of human pulmonary tuberculosis (1, 2) and certain infections of the urinary tract (3). The present study was stimulated by the rather broad variations in the reported tolerance of cycloserine by different subjects (1–6) and the recognition that differences in tolerance sometimes reflect differences in physiologic disposition of a drug. The investigation seemed particularly pertinent in view of the striking discrepancies in previous observations (7, 8) on the excretion of cycloserine and the recognition that such discrepancies might rest on the use of unsatisfactory assay procedures.

MATERIALS AND METHODS

Recovery of drug in urine: The urinary excretion of cycloserine[3] after oral administration was studied in 5 normal human subjects. The drug, in gelatin capsules, was given as a single dose of 4 mg. per kg. along with a glass of water. The excretion of cycloserine was also studied in 2 subjects who received the compound both orally and intramuscularly in doses of 2 mg. per kg. of body weight. For the latter study the drug was administered as a solution of 50 mg. of cycloserine per ml. in 2.5 per cent sodium carbonate, sterilized by filtration through sintered glass. The oral dose was diluted with 300 ml. of water.

In both studies a twenty-four-hour control specimen of urine was collected immediately prior to drug administration. Fractional collections were made at successive intervals of two, four, four, six, and eight hours during the first twenty-four hours after dosage and at twelve-hour intervals for the next two days. In order to prevent possible decomposition of cycloserine at an acid pH, urine samples were voided over sufficient sodium carbonate to give a one per cent solution and were stored at 4° to 6°C. No restrictions were placed on the subjects during the collection period other than that they refrain from ingesting other drugs or alcoholic beverages.

A modification of the method of Jones (9), involving analysis of urine samples diluted 1:5 with one per cent sodium carbonate, was used in determining the cycloserine content of urine. This dilution step was essential for quantitative recoveries of this drug. Previous studies in this laboratory, as well as observations by Jones (9), had shown that undiluted urine of normal humans contains substances which interfere with the formation of the cycloserine-nitritopentacyanoferroate complex and that the urinary content of these substances varies both in different subjects and in the same individual. In some urine specimens as much as a 39 per cent depression in color development has been observed. Systematic studies showed that these suppressing effects could be reduced to an insignificant level (less than 3 per cent) by diluting the urine 1:5 with one per cent sodium carbonate.

[1] From The Christ Hospital Institute of Medical Research, Cincinnati, Ohio.

[2] This investigation was supported, in part, by a research grant (E 509) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

[3] Kindly supplied by Eli Lilly and Company.

Such dilutions were made on all urine specimens collected post treatment after it was determined that the dilution step essentially eliminated the effect of interfering substances contained in pretreatment control specimens.

The nature of the cycloserine metabolites excreted in the urine of one subject was studied, using paper chromatographic procedures similar to those described previously (10). Aliquots of treatment urine, containing 250 γ of cycloserine per ml., were applied in amounts varying from 0.01 to 0.05 ml.

Clearance of cycloserine from plasma: In the 2 subjects who excreted cycloserine most rapidly, simultaneous measurements were made of the disappearance of the drug from the plasma and its recovery in the urine. The daily dose of 8 mg. per kg. was divided into three equal fractions and was administered orally in gelatin capsules at 7:00 a.m., 3:00 p.m., and 11:00 p.m. Two hours after the seventh and last dose, the concentration of cycloserine in the plasma of each subject was determined. Plasma cycloserine concentrations were again measured four and eight hours after the last dose. Urine fractions corresponding to the time intervals between successive blood samples were collected and assayed for cycloserine as described above. The method used for determining the concentration of cycloserine in plasma was an adaptation of the whole blood procedure of Jones (11). Five per cent trichloroacetic acid was used instead of tungstic acid to prepare a protein-free filtrate; aliquots of the filtrates were assayed according to the later method described by Jones (9).

RESULTS

The data presented in table 1 show that the total urinary excretion of cycloserine was remarkably similar in all 5 subjects who received the 4 mg. per kg. dose, varying from 60.6 to 65.9 per cent of the administered drug in a seventy-two-hour period. The first twelve-hour output accounted for approximately 50 per cent of the total cycloserine elimination; the first twenty-four hours, for approximately 80 per cent.

Although the rate of cycloserine excretion varied considerably among the individual subjects (*cf.* table 2), the highest mean excretion rate, approximately 125 γ per kg. per hour, occurred two to six hours after drug administration.

The data in table 3 show that in 2 subjects, at least, excretion of cycloserine was essentially identical following oral or intramuscular administration. This finding points to completeness of absorption of the drug from the gastrointestinal

TABLE 1

CUMULATIVE URINARY EXCRETION OF CYCLOSERINE*

Subject	Sex	Body Weight	Per Cent of Dose Recovered					
			Hours After Oral Administration					
			2	6	12	24	48	72
		kg.						
MA	M	79.5	6.1	16.2	25.0	46.6	58.2	62.6
GB	M	75.4	4.7	17.0	35.5	55.3	63.5	65.9
AG	M	79.5	6.1	18.4	29.0	39.6	57.0	62.3
HH	F	62.8	8.1	22.8	41.4	59.1	64.4	64.4
RS	M	84.5	3.3	16.0	28.2	44.7	56.6	60.6
Mean			5.7	18.1	31.8	49.1	59.9	63.2

* Dose: 4 mg. per kg. of body weight.

TABLE 2

RATE* OF URINARY EXCRETION OF CYCLOSERINE AFTER SINGLE ORAL DOSE OF 4 MG. PER KG.

Subject	Body Weight	Hours After Dose				
		0-2	2-6	6-10	10-16	16-24
	kg.					
MA	79.5	122.0	101.0	67.6	42.0	86.5
GB	75.4	94.2	123.5	139.1	100.5	46.5
AG	79.5	122.5	123.3	79.0	53.2	26.1
HH	62.8	161.0	147.1	138.5	94.0	41.0
RS	84.5	66.2	127.0	88.3	72.2	45.4
Mean		113.2	124.4	102.5	72.4	49.1

* Expressed as γ per kg. of body weight per hour.

TABLE 3

CUMULATIVE URINARY EXCRETION OF CYCLOSERINE*

Subject	Body Weight	Route of Administration	Per Cent of Dose Recovered				
			Hours After Administration				
			6	12	24	48	72
	kg.						
GB	75.4	Oral	17.2	33.5	50.8	64.4	66.9
GB	75.4	Intramuscular	19.1	33.6	49.3	60.2	63.6
GC	87.5	Oral	20.0	31.9	50.8	63.2	66.9
GC	87.5	Intramuscular	22.9	37.8	51.2	64.5	65.9

* Dose: 2 mg. per kg. of body weight.

tract and to metabolism within the body. It should also be noted (*cf.* tables 1 and 3) that recoveries of cycloserine in subject *G. B.* on the 2 and 4 mg. per kg. doses were nearly identical, suggesting that, at least within this range, metabolism and excretion of the drug are independent of the dosage.

The results of two-dimensional chromatographic studies suggest strongly that only one nitritopentacyanoferroate-reacting substance was present in the urine of subjects who received cycloserine. This substance was indistinguishable from the parent drug.

The results of the study on the clearance of cycloserine from the plasma are summarized in figure 1 and table 4. The data in figure 1 suggest that the peak concentration of cycloserine was obtained within two hours after administration and declined logarithmically thereafter. The data in both figure 1 and table 4 point to differences in the disposition of the drug in these subjects. Thus the half-life, i.e., the time required for the drug concentration in the plasma to reach a value one-half that determined previously, was estimated as eight hours for

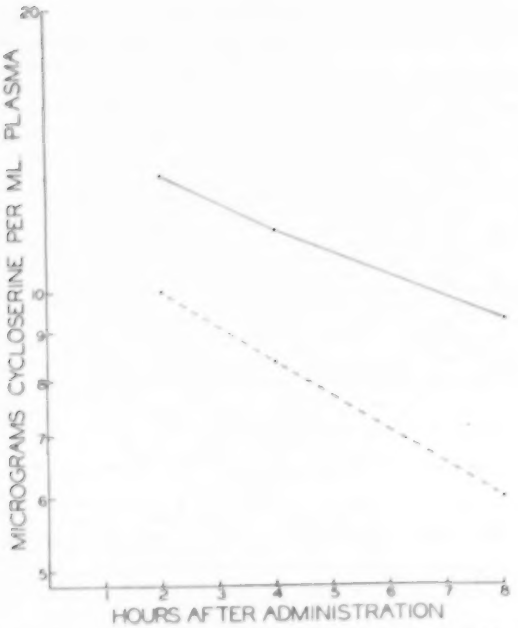

FIG. 1. Cycloserine concentrations in the plasma of two human subjects after repeated oral dosage of 8 mg. per kg. per day.
———: Subject *GB*, body weight 75.4 kg.
– – –: Subject *HH*, body weight 62.8 kg.

TABLE 4

RATE* OF URINARY EXCRETION OF CYCLOSERINE AFTER REPEATED DOSE REGIMEN OF 8 MG. PER KG. PER DAY

Subject	Body Weight	Hours After Last Oral Dose			
		0-2	2-4	4-8	8-24
	kg.				
GB	75.4	153	192	159	117
HH	62.8	570	302	282	78

* Expressed as γ per kg. of body weight per hour.

subject *H. H.* and twelve hours for subject *G. B.* Cycloserine clearance values were 41 and 22 ml. of blood per minute for the respective subjects.

DISCUSSION

The results of the present study indicate that the total urinary excretion of cycloserine following oral administration to a group of human subjects was remarkably uniform. Individual values were found to deviate from the mean of

63 per cent recovery by less than 3 per cent. The results of the present excretion study differ markedly from those reported earlier by other investigators (7, 8). Welch and associates (7) found that the mean cumulative excretion for subjects who received 250 or 500 mg. orally was 13 and 17 per cent, respectively, of the total dose. Nair and associates (8), on the other hand, reported twenty-four-hour recoveries up to 92 per cent of a single dose of 500 mg.[4]

The reasons for these marked discrepancies are difficult to ascertain, but the following possibilities may be considered: (*a*) intrabladder decomposition of cycloserine; (*b*) decomposition of cycloserine during storage of urine specimens; and (*c*) presence of nonspecific substances in urine which interfere with the method of analysis. Experiments carried out in this laboratory suggest that decomposition of cycloserine either in the bladder or in stored urine probably plays a very minor role in the over-all recovery of the drug. Dilute solutions (120 γ per ml.) of cycloserine prepared in sterile urine (pH 5.2) were found to be relatively stable even when incubated for twelve hours at 37°C. The observed decomposition was less than 15 per cent. Dilute aqueous solutions of cycloserine (1 mg. per ml. or less), in contrast to concentrated solutions (50 mg. per ml.) (10), have been found to decompose slightly at room temperature; however, it is doubtful that the storage of urine specimens at room temperature for a short time prior to analysis would account for the marked differences observed.

The most probable explanation for these observed discrepancies is the presence in urine of substances which interfere with the formation of the color complex. Undiluted control urine samples, containing sufficiently high concentrations of these substances to effect a diminution in color of 39 per cent, have been encountered in this laboratory. It was found that a 1:5 dilution of the specimen with 1 per cent sodium carbonate solution would reduce this suppressing effect to an insignificant level. Although the nature of these substances is unknown, it seems entirely possible that urinary metabolites derived from substances ingested in the diet or as medications might introduce errors even larger than those found in the present investigation, if undiluted urines were used for assay.

The extent of urinary excretion of cycloserine after intramuscular administration suggested that an appreciable amount of the drug was metabolized by the human subject, as had been found previously in the monkey (10). Furthermore, the observation that nearly identical drug excretion values obtained after either intramuscular or oral administration suggested that absorption from the gastrointestinal tract was both rapid and complete.

Results of paper chromatographic studies on urine samples from a human subject receiving cycloserine by mouth indicate the presence of a single substance which reacts with nitroprusside and the identity of this material to the parent

[4] In a recent report, published subsequent to completion of the present investigation, Renzetti and co-workers (13) summarized data on the urinary excretion of cycloserine in a group of eight patients who received repeated daily doses of 1 gm. In these subjects, the daily excretion averaged between 0.30 and 0.68 gm., i.e., from 30 to 68 per cent of the daily dose. These data are more nearly in agreement with those of the present study than observations reported heretofore (7, 8).

drug. These findings parallel closely those obtained in similar studies on the monkey (10).

The plasma cycloserine curves shown in figure 1 indicate that the maximal plasma concentration was reached within two hours after the last dose and that from this peak the concentration in the plasma diminished nearly logarithmically for eight hours to reach a level of 37 to 49 per cent lower than the peak concentration. This finding does not support the conclusion of Nair and associates (8) that the cycloserine concentration in the blood remained at the peak (two-hour) level for eight hours after administration, but does agree with the observation of Welch and associates (7).

Evidence presented in the current report suggests that administered cycloserine may distribute itself uniformly in the total body water compartment. Metabolism and urinary excretion occurring during the first two hours after administration can account for only a small fraction of the total drug disposition; consequently, the low plasma concentrations obtained can be explained either by assuming distribution of the drug in a large fluid compartment (30 to 35 liters) or by tissue binding. The close agreement between the plasma concentration of cycloserine and that in cerebrospinal fluid (8) and milk (12), together with the reported ubiquitousness of ingested cycloserine in various other body fluids (8, 12), lends support to the supposition that this drug is distributed chiefly in the body water compartment. Since the known water solubility of the drug precludes saturation of this fluid compartment by the administration of even the highest therapeutic doses, the plasma concentrations resulting from a chronic dosage regimen are probably dependent solely on the body water volume and the excretion-metabolism pattern of the individual. As a result, the plasma concentration tends to stabilize at that level at which the total drug disposition becomes equal to the dose administered. Subject *G. B.*, for example, was found to maintain a peak plasma level of 14 to 17 γ per ml. on a dose of 8 mg. per kg. per day. Subject *H. H.*, because of a higher clearance rate, would probably stabilize at a somewhat lower level.

The present data, showing that a major portion of a given dose of cycloserine is eliminated by the kidney, may present a contraindication to the use of this drug in patients with renal impairment.

SUMMARY

The excretion and metabolism of cycloserine, administered orally and intramuscularly to the human subject, have been studied. Approximately 64 per cent of the dose given by oral administration was excreted in the urine within seventy-two hours, the first twelve hours accounting for approximately 50 per cent of the total cycloserine elimination. The period of maximal excretion rate occurred during the interval of two to six hours after administration. Renal excretion of cycloserine following parenteral administration was not significantly different from that observed after the same dose was given orally.

Excretion data suggested that approximately 35 per cent of a given dose was metabolized to unknown substances. Chromatographic studies indicated that

urine samples from the treated human subject contained only one compound, cycloserine, which reacts with the nitroprusside reagent.

The results of experiments described in this report suggest that ingested cycloserine becomes uniformly distributed in the total body water compartment and that the plasma concentrations resulting from repeated dosage are dependent on the volume of this compartment and the excretion-metabolism pattern peculiar to the individual.

SUMARIO

La Disposición Fisiológica de la Cicloserina en los Sujetos Humanos

Versa este estudio sobre la excreción y el metabolismo de la cicloserina, administrada oral e intramuscularmente a sujetos humanos. Aproximadamente 64 por ciento de la droga administrada oralmente fué excretada en la orina en término de setenta y dos horas, correspondiendo a las primeras doce horas aproximadamente 50 por ciento de la eliminación total de cicloserina. El período de excreción máxima fué durante el plazo de dos a seis horas consecutivo a la administración. La excreción renal de cicloserina a continuación de la administración parentérica no discrepó mayor cosa de la observada después de administrar la misma dosis por vía bucal.

Los datos de excreción sugirieron que aproximadamente 35 por ciento de una dosis dada se metabolizaba en substancias desconocidas. Estudios cromatográficos indicaron que ejemplares de orina del sujeto tratado no contenían más que un compuesto, cicloserina, que reaccionara con el reactivo de nitroprusiato.

Los resultados de los experimentos descritos en esta comunicación sugieren que la cicloserina ingerida se distribuye uniformemente en el compartimiento del agua total del organismo y que las concentraciones plasmáticas debidas a dosis repetidas corresponden al volumen de este compartimiento y al patrón de excreción-metabolismo peculiares del sujeto dado.

RESUME

Répartition physiologique de la cyclosérine chez l'homme

L'excrétion et le métabolisme de la cyclosérine, administrée par les voies orale et intramusculaire chez l'homme, ont été étudiées. Approximativement 64% de la dose administrée par voie orale fut excrétée dans l'urine en soixante-douze heures; c'est durant les douze premières heures qu'approximativement 50% de la dose totale de cyclosérine furent éliminés. La période où le taux de l'élimination atteignait son maximum se situait entre deux à six heures après l'administration. L'excrétion rénale de la cyclosérine à la suite de l'administration parentérale ne différait pas sensiblement de celle observée lorsque la même était donnée oralement.

Les données relatives à l'excrétion suggéraient qu'approximativement 35% d'une dose déterminée était métabolisée en substances inconnues. Les études chromatographiques indiquaient que les urines prélevées chez l'homme traité par la cyclosérine ne renfermaient qu'un seul composé, la cyclosérine, qui donne une réaction avec le nitroprussiate.

Les résultats de l'expérimentation décrite dans ce rapport suggèrent que la cyclosérine ingérée se répartit uniformément dans la totalité des liquides extracellulaires et que les concentrations plasmatiques résultant de l'administration répétée dépendent du volume du liquide extracellulaire et des modalités du métabolisme d'excrétion particulier chez le sujet.

Acknowledgment

The cooperation of those who served as subjects for this study and the helpful criticism of Dr. L. H. Schmidt are gratefully acknowledged.

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THE SURGICAL TREATMENT OF PULMONARY TUBERCULOSIS COMPLICATING DIABETES MELLITUS[1]

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INTRODUCTION

Before insulin was available, pulmonary tuberculosis was a very common cause of death in diabetics. Tuberculosis was found by Griesinger (1) in 42 per cent of 250, and by Windle (2) in 50 per cent of 333 autopsies on diabetics, whereas Montgomery (3) reported that 24 per cent of autopsies on diabetics showed evidence of active pulmonary tuberculosis. More recently, Boucot and associates (4) found a prevalence of tuberculosis of 8.4 per cent among 3,106 diabetic patients in Philadelphia, which was twice as high as the prevalence in a group of apparently healthy industrial workers. The *Lancet* (5) suggested that selective factors in addition to those considered by the writers may have been present. In the same survey, diabetics of less than forty years of age had a prevalence of active disease of 5.3 per cent, compared with 1.7 per cent for those more than forty; and in the younger group the prevalence of active disease was directly related to the duration of the diabetes. The prevalence of active tuberculosis was also higher (5.3 per cent) in diabetics who received more than 40 units of insulin per day than in those who received less, or none (1.5 per cent).

The evidently serious prognosis of this combination of diseases was indicated as late as 1946 by Poulsen's finding (6) that 91 of 100 patients with diabetes with pulmonary tuberculosis had died after an observation period of five years. Banyai and co-workers (7), in a comprehensive review of the problem of tuberculosis in diabetics in the pre-chemotherapy era, studied 115 tuberculous diabetic patients. Of these 115 patients, on discharge from the sanatorium, 47 per cent with moderately advanced disease were classified as having arrested, quiescent, or improved tuberculosis; and in 53 per cent the disease was unimproved or the patient had died. Of the far advanced group 14.5 per cent had apparently arrested, quiescent, or improved disease; whereas in 85.4 per cent, the disease had remained unimproved or had been fatal. The writers comment that these results are less favorable than those recorded for similar patients without diabetes. They found that patients whose diabetes was controlled to the extent that there was only slight glycosuria, and hyperglycemia not exceeding 200 mg. per 100 ml., did as well as those whose blood sugar was maintained at a practically normal level.

On the other hand McKean and associates (8), reviewing 117 patients with the two diseases, found that the mortality paralleled closely that of patients with pulmonary tuberculosis alone. Himsworth (9), describing his experience of 15 patients with pulmonary tuberculosis from a series of 230 consecutive cases of diabetes, expounds the proposition that:

[1] From the High Carley Hospital, Ulverston, England.

Diabetes predisposes to the development of tuberculosis, and when this predisposition is removed by treatment the diabetic is in a relatively stronger position than the non-diabetic, whose general resistance was so poor that he developed a similar lesion although not under the handicap of diabetes.

He endorses Root's (10) suggestion that

. . . the prognosis in pulmonary tuberculosis in diabetics may be better than in non-diabetics, if they are discovered at relatively the same stage of tuberculosis.

It has been shown that the incidence of tuberculosis in diabetics declined following the discovery of insulin; and with the introduction of effective antimicrobial treatment, the position can clearly be expected to improve still further.

Relatively little has been written regarding the surgical treatment of pulmonary tuberculosis in diabetics. Thosteson and McKean (11) describe eleven such patients who were treated by thoracoplasty, the tuberculosis being advanced in all cases. There were no immediate postoperative deaths; one patient died four and one-half months later from respiratory insufficiency, one died two years later from myocardial infarction, and one died ten months after operation from massive hemoptysis; in seven patients the disease was arrested.

In the series of Thosteson and McKean, the diabetes was mild in four patients and moderately severe or severe in seven; after operation it became more severe in six patients, as shown by increased insulin requirements; three patients needed less insulin, and two required it for only a few days after operation. The writers conclude that diabetics tolerate extensive chest surgery satisfactorily, but that, in spite of improved control of the infection, there is not necessarily an improvement in the diabetes. The fatality rate observed (27 per cent) does not differ greatly from that quoted by O'Brien and associates (12) who reported a rate of 19 per cent of 511 nondiabetic patients treated by thoracoplasty.

Thompson (13) described a thoracoplasty in a fairly severe diabetic, the operation passing uneventfully and being followed by a substantial reduction in insulin requirements; and Wacker (14) and Källqvist (15) each describe single cases successfully treated by resection. Lebon and co-workers (16) describe a patient treated by pneumonectomy who suffered a relapse and died two years after operation.

The present report records the largely favorable results in a consecutive series of 11 patients with severe diabetes, i.e., requiring at least 40 units of insulin daily, whose pulmonary tuberculosis was treated by major surgery.

OBSERVATIONS

The series was composed of 8 men and 3 women who varied in age from seventeen to forty-four years at the time of the diagnosis of tuberculosis. In all cases the sputum or stomach washings contained tubercle bacilli, and cavitation was present. Using the classification according to roentgenographic extent of

[1] *Class I:* not more than one zone in one lung involved.
Class II: unilateral or bilateral disease involving two or three zones.
Class III: four or more zones involved.

disease described by Foster-Carter and associates (16),[2] one patient falls into *Class I*, 4 into *Class II*, and 6 patients into *Class III*. The diabetes required from a minimum of 45 to a maximum of 208 units of insulin daily at the time of admission to the hospital.

Seven patients were treated by thoracoplasty, 3 by resection, and one by decortication for tuberculous empyema. They have been followed for periods from ten months to four years and three months.

Case Reports[3]

Case 1. Thoracoplasty. G. I., born in 1924, is the only patient in the series in whom the diagnosis of tuberculosis preceded that of diabetes. In 1945 he was found to have tuberculous infiltration in the upper half of the right lung, for which he was kept under observation as an outpatient, remaining quite well and at work. In 1948 he first developed symptoms of diabetes, which was controlled with insulin; he then remained well until a few months before admission to the hospital in January, 1951, when he developed increasing lassitude, right-sided chest pain, and the diabetes became unstable. A roentgenogram showed extension of the disease in the right upper lobe with cavitation, and on admission gastric washings were positive for tubercle bacilli. In spite of 108-100 units of insulin daily, the diabetes was uncontrolled, with severe ketosis. It was stabilized on bed rest and after six months there was some roentgenographic improvement, and the patient's insulin requirements had dropped to 88-84 units daily; at no time did he have chemotherapy. In August, 1951, the patient underwent a one-stage three and one-half-rib right thoracoplasty followed by air refills to the Semb's space, and recovery from operation was uneventful. He has since remained fit and at work as a clerk, apart from a transient pleurisy in October, 1955, which cleared up completely in two weeks. The insulin requirements have decreased to XXVIII/36-40 units daily, and the sputum has been negative for tubercle bacilli on microscopy and culture since operation.

Case 2. Thoracoplasty. G. M., born in 1917, was discharged from the army in 1945 with severe diabetes of recent origin. Following institution of treatment he remained well and at work until he developed acute pneumonic symptoms in 1949. The sputum was found to contain tubercle bacilli, and his chest roentgenogram showed a collapsed right upper lobe with multiple cavities, together with bronchogenic spreads in both mid zones. On admission to the hospital in March, 1951, he was having XXXVI/40 units of insulin daily, but the diabetes was unstable, with severe ketosis. This was gradually controlled with bed rest and 112-56 units of insulin daily; after six months there was appreciable roentgenographic improvement. In September, 1951, a right six-rib thoracoplasty, followed for three months by air refills to the Semb's space, was carried out. Although tubercle bacilli were found in the sputum on several occasions, cavity closure was obtained after operation and his insulin requirements came down to 40-40 units daily.

Other than PAS, given alone for approximately three weeks before admission, the patient has had no chemotherapy. He has remained well and has been working as a bank clerk; the sputum has been consistently negative for tubercle bacilli since he left the hospital.

[3] Soluble insulin is represented by arabic numerals in one, two, or three daily doses as indicated; zinc-protamine insulin, by roman numerals. Roman and arabic numerals occurring together, e.g., XXXVI/40 indicate that the two insulins are given at the same time. Additional arabic numerals indicate an extra dose of soluble insulin taken later in the day, e.g., XXVIII/36-40.

Case 3. Thoracoplasty. Miss B. P., born in 1933, has had insulin since diabetes was discovered in 1943. Pulmonary tuberculosis was diagnosed in 1949 following an hemoptysis; at that time she had acute disease in the right upper lobe with partial collapse and cavitation and a limited spread of disease in the mid zone; the left lung was normal. On admission to the hospital in December, 1945, the diabetes was unstable, with constant glycosuria and frequent ketone bodies. The patient was treated with rest and insulin, 32-30-32 units daily, but had no antituberculous chemotherapy at any time. Her condition steadily improved and the diabetes stabilized, but the sputum remained positive for tubercle bacilli. A right artificial pneumothorax having failed, she was submitted to a two-stage seven-rib thoracoplasty without apicolysis in November and December, 1951, and her convalescence was uneventful.

At the time of discharge she was having XXIV/24 units of insulin daily, and she has remained well since. A pregnancy in 1954 was uneventful.

Case 4. Thoracoplasty. P. T. was born in 1896. Diabetes was diagnosed in 1933, and a year later he was found to have tuberculous infiltration in the upper zones of both lungs, and the sputum was positive for tubercle bacilli. He required 20-10-15 units of soluble insulin daily and remained in good health, but the tuberculous disease in the left upper lobe very slowly progressed and in 1942 was found to be cavitating. After a period of sanatorium rest, he remained fairly well until 1951, when the cavity in the left upper lobe enlarged and tubercle bacilli were again present in the sputum. In January, 1952, when the patient was 56 years old, a left two-stage seven-rib thoracoplasty was performed and was followed by apparently satisfactory control of the disease; immediately after operation his daily insulin requirements rose to 80-50-30 units, but quickly settled to 20-10-15 units. Streptomycin-PAS was given, for four weeks only, over the operative period. He remained well, with no tubercle bacilli in the sputum, and worked as a teacher until, in September, 1955, the sputum was found to contain tubercle bacilli, although there was no roentgenographic change. Since then he has been on continuous isoniazid-PAS, and remains free of symptoms, taking 14-12-8 units of insulin daily; the urine contains only an occasional trace of sugar.

It is noteworthy that there was never any evidence of acute deterioration, in spite of persistent cavitation for ten years before operation.

Case 5. Thoracoplasty. W. F. S., born in 1921, was discharged from the army in 1944 with diabetes of recent origin. His symptoms were controlled with insulin, and he remained well until 1951, when he developed right chest pain. A chest roentgenogram showed an atelectatic right upper lobe with a 2 cm. cavity and scattered infiltration in the right mid zone and in the upper and mid zones in the left lung. On admission to the hospital in September, 1951, his diabetes was unstable, with severe ketosis, heavy glycosuria, and occasional hypoglycemic attacks. Following eight months' bed rest and streptomycin-PAS therapy, there was satisfactory clearing of the scattered disease and contraction of the right upper lobe cavity. The diabetes became stable after the insulin had been increased from XLVI/36 to 64-32-20 units daily. In May, 1952, the patient underwent a two-stage five and one-half-rib right thoracoplasty with apicolysis, followed by Semb's space air refills for three months. He was the only patient in the series to have any postoperative septic complication; he had a low-grade wound infection, which disappeared after a week of penicillin therapy, but which had produced a transient but marked ketosis. During the ensuing weeks the patient had several severe hypoglycemic attacks which required a steady reduction in insulin, eventually to XXXIV/40 units daily.

He has since remained well, with sputum negative for tubercle bacilli on microscopy and culture, and is working as a clerk.

Case 6. Multiple localized resections. D. R., a male, born in 1932, has had regular insulin for severe diabetes since 1944. In 1949 a routine roentgenogram showed several round homogeneous opacities, up to 4 cm. in diameter, in each lung, and the sputum was positive for tubercle bacilli. He was treated by left artificial pneumothorax, and later by pneumoperitoneum, with a short course of streptomycin-PAS, and remained well until he was readmitted to the hospital in July, 1954.

At this time his general condition was fair; the diabetes was unstable, with occasional ketosis and constant heavy glycosuria in spite of 50-40 units of insulin daily. His roentgenogram showed a large thick-walled cavity in the right mid zone, a solid focus in the right apex, and several solid lesions in the left upper and mid zones, one of which had cavitated. After admission his tubercle bacilli were found to be resistant to isoniazid, and he was treated with streptomycin-PAS throughout his stay in the hospital, together with insulin, 40-40-40 units daily; the ketosis disappeared and the glycosuria was greatly reduced. In October, 1954, the right lower lobe apex was resected and found to contain no less than four cavities; and in April, 1955, four solid lesions were removed by wedge resection from the left lung, one small lesion being left in the upper lobe. For a few days after each operation he had marked ketosis, but his convalescence was uneventful except for occasional hypoglycemic reactions as his insulin requirements grew less.

Since discharge from the hospital in September, 1955, the patient has remained well, with sputum negative for tubercle bacilli on microscopy and culture. He takes XLVI/40 units of insulin daily, and works full-time as a draftsman.

Case 7. Thoracoplasty. G. M., born in 1912, was found to have diabetes in 1938, and has been treated with insulin since that time. Tuberculosis was diagnosed in 1945 following an hemoptysis. At the time of diagnosis there was atelectasis of the left upper lobe with cavitation; the patient was treated by left artificial pneumothorax, which was maintained for six years with contraselective collapse. During this period he remained in quite good health but, following re-expansion of the lung in 1952, the disease spread to the right mid and left lower zones, for which he was treated for three months with streptomycin-isoniazid. On admission to the hospital in September, 1954, the diabetes was quite unstable, with persistent heavy glycosuria and severe ketosis, but after two months on insulin, 60-20-20 units daily, and streptomycin-isoniazid, the diabetes was fully controlled. The sputum remained positive for tubercle bacilli and tomography confirmed a persisting cavity in the left apex. Consequently, in December, 1954, the patient had a two-stage six-rib left thoracoplasty with apicolysis; convalescence was quite uneventful.

At the time of discharge in June, 1955, his insulin requirements had dropped to XXXVI/46 units daily, the diabetes was stable, and he was fully active, symptom free, and had no tubercle bacilli in the sputum.

Case 8. Pneumonectomy. Mrs. M. E., born in 1923, was found to have diabetes in 1944, and has been treated with insulin since. In 1949 a chest roentgenogram showed an almost completely destroyed left lung, with gross cavitation, and limited infiltration in the right upper lobe. At that time the patient was treated with 100 gm. of streptomycin alone, with only temporary improvement, and later with isoniazid alone. When admitted to the hospital in August, 1954, her diabetes was unstable, with heavy glycosuria and occasional ketone bodies; she was having 40-20-40 units of insulin daily, and the fasting blood sugar was more than 300 mg. per 100 ml. Her tubercle bacilli were significantly resistant to streptomycin and isoniazid. The patient was treated with viomycin, 2 gm. twice weekly, and with PAS, with considerable improvement in her general condition, roentgenographic contraction of the disease on the right side, and control of the ketosis. Before operation the insulin was reduced to 40-12-28 units daily. The left lung was removed on January 10, 1955, and the postoperative course was uneventful. During convalescence the viomycin and PAS were con-

tinued for a further six months, and the insulin requirements dropped to XXVI/32 units daily. During early convalescence the patient had numerous hypoglycemic attacks, but has remained well and free from symptoms since discharge from the hospital in July, 1955. The fasting blood sugar on October 21, 1955, was 58 mg. per 100 ml.

Case 9. Decortication. L. M., born in 1915, has been having insulin treatment since diabetes was discovered in 1935; pulmonary tuberculosis was diagnosed in 1949. At that time his roentgenogram showed a 4 cm. tension cavity in the right upper lobe, with partial atelectasis. A right artificial pneumothorax was induced, and the patient rapidly developed a large effusion, which contained tubercle bacilli and progressed to frank pus. Shortly after this he developed a cavitating lesion in the left upper lobe which has persisted since. Between 1950 and 1954 he had several short courses of streptomycin alone, and later received streptomycin with PAS and isoniazid. Early in 1954 the patient developed a right tuberculous epididymitis, which was treated by excision; and at the same time he developed for the first time a bronchopleural fistula. He was admitted to the hospital in November, 1954, in the hope that, if the empyema could be excised and the fistula closed, it might be possible to treat the lesion in the left upper lobe.

On admission the diabetes was unstable, with constant glycosuria and ketosis; insulin was therefore increased from 40-20 to 50-30-30 units, and daily streptomycin-isoniazid-PAS was instituted, with control of the ketosis. In February, 1955, decortication of the right lung was carried out, but the lung failed to expand, the fistula persisted, and subsequently permanent open drainage was instituted. In May, 1955, tubercle bacilli were found for the first time to be resistant to PAS and streptomycin, but susceptible to viomycin and isoniazid, which drugs have been continued to date. His sputum remained positive for tubercle bacilli, but he was free of ketosis and in good general condition, and was discharged to live quietly at home. The insulin requirements of this patient had dropped to 56-18 units daily. In December, 1955, the empyema became secondarily infected with a penicillin-resistant staphylococcus; the diabetes again became unstable, but was controlled after treatment with chlortetracycline. The disease in the left upper lobe has improved meanwhile, but the patient has refused further surgical treatment.

This is the only unsatisfactory case in the series, the failure being evidently due to empyema complicating an ill-advised artificial pneumothorax. Nevertheless, in spite of a large tuberculous empyema, the patient remained fairly well for four years before developing a bronchopleural fistula.

Case 10. Thoracoplasty. Mrs. M. B., born in 1909, has been under treatment for diabetes since 1934; in 1946 she developed pernicious anemia, which has since been controlled by liver and later by vitamin B_{12}. Pulmonary tuberculosis was diagnosed in January, 1953, several previous routine chest roentgenograms having been normal. A large cavity was present in the left upper lobe with bronchogenic spread of disease to the right upper and left mid and lower lobes. Following three months of chemotherapy the new disease cleared but the cavity persisted; and a year later, in June, 1954, the patient's condition again deteriorated. On admission to the hospital in January, 1955, after six months of daily treatment with isoniazid-streptomycin, there was still a 2 cm. cavity in the left upper lobe, and the sputum remained positive for tubercle bacilli on culture. She had severe ketosis, and the urine was constantly heavily loaded with sugar; the insulin was increased from 44-16 to 56-22-32 units daily, with control of the ketosis, although the heavy glycosuria continued and the blood sugar remained over 300 mg. per 100 ml. The patient proved difficult to educate in the matter of diet. After five months of postural rest and continued chemotherapy, there was substantial reduction of the left apical cavity. A left two-stage six-rib thoracoplasty was carried out in May, 1955, the postoperative course being uneventful. By September the fasting blood sugar had dropped to 120 mg. per 100 ml., and the insulin dosage had been reduced to XXX/34 units daily.

The patient has since remained well and the sputum has been negative for tubercle bacilli on microscopy and culture.

Case 11. Segmental resection. A. R. P., born in 1923, developed diabetes, requiring insulin, in 1949. Tuberculosis was diagnosed in January, 1954, following an hemoptysis, at which time there was a 4 cm. thick-walled cavity in the right lower lobe apex. He had been treated with continuous multiple-drug chemotherapy from February, 1954, and the cavity had contracted to a diameter of 1 cm. On admission to the hospital the patient was having XX/56-12 units of insulin daily, with occasional ketosis and varying glycosuria. The diabetes stabilized on 50-20-20 units of soluble insulin, and in June, 1955, the right lower lobe apex was removed; the patient's insulin requirements increased to a maximum of 64-34-34 units but there was no ketosis. The postoperative course was uneventful, and he has remained well since on 64-28 units daily, with sputum negative for tubercle bacilli on microscopy and culture.

DISCUSSION

In one patient (*Case 1*) tuberculosis was diagnosed three years before the onset of symptoms of diabetes; in the remaining 10 patients, the diabetes preceded the discovery of tuberculosis by periods ranging from one to eighteen years (table 1).

Dubos (17) quotes experimental evidence that ketosis is probably the most important factor in starvation which causes activation of tuberculous foci; it seems reasonable to infer that this may also be the case in diabetes. All of the patients in the present series had some degree of ketosis, as well as hyperglycemia, at the time of admission; treatment was aimed primarily at control of the ketosis. Soluble insulin was given in divided (usually three) daily doses, and in no instance was any long-acting insulin used until some weeks after operation. No attempt was made to maintain the blood sugar at normal levels, but in practice the patients were free of ketosis, with only occasional glycosuria, within a few weeks of starting treatment. As would be expected, control was effected most rapidly in patients who received chemotherapy for recently activated disease.

The operations were planned to start at 10 a.m., the aim being to send the patients to the operating theater with a moderate glycosuria. To this end a light breakfast (tea and toast) was given at 7 a.m., with some 20 units of soluble insulin; additional glucose by mouth was given if the urine was sugar free. Im-

TABLE 1

TIME OF ONSET OF TUBERCULOSIS IN RELATION TO DIABETES

Diabetes Preceded Tuberculosis in Ten of the Eleven Patients

Patient Number	1	2	3	4	5	6	7	8	9	10	11
Extent of disease*	2	2	2	3	3	2	3	3	3	1	3
Age at diagnosis of pulmonary tuberculosis	21	32	17	37	30	18	33	26	44	31	34
Age at diagnosis of diabetes	24	28	10	36	23	13	26	21	26	26	20
Interval (years)	−3	4	7	1	7	5	7	5	18	5	14

* Classified according to Foster-Carter and associates (17).

TABLE 2

INSULIN REQUIREMENTS

Patient Number.	1	2	3	4	5	6	7	8	9	10	11
On admission	208	76	82	45	82	90	94	100	56	90	60
Immediately after operation	172	168	94	180	116	120	100	80	108	132	110
On completion of treatment	120	80	48	34	74	86	82	60	64	92	74

The total daily dose of insulin, whether soluble or zinc-protamine, in single or divided doses, is given as a single figure, to give a general indication of the severity of the diabetes and its behavior at different stages of treatment.

Eight patients required a considerable temporary increase in insulin immediately after operation. On completing treatment, 5 required substantially less insulin than before operation; in 5 there was little change in requirements; one (*Case 11*) needed an increase, due to secondary infection of his empyema, some months after discharge.

mediately on recovering consciousness, three-hour urine testing was started (in only one instance was catheterization necessary); and soluble insulin, covered by glucose by mouth, was given according to the degree of glycosuria. The three-hour regimen was maintained for one week, solid foods being gradually introduced from the third day. Intravenous fluids were not required.

Once normal feeding was re-established, rapid reduction in insulin was necessary, to the preoperative level or less (table 2). During this period 3 patients suffered considerable discomfort from hypoglycemic attacks. Seven patients were stabilized on a single daily injection of long-acting insulin before discharge from the hospital.

Four patients (*Cases 1* to *4*) had no effective chemotherapy, i.e., less than four weeks in all, while 7 had antimicrobial treatment for periods from eight to thirty-two months before operation. Strains of tubercle bacilli significantly resistant to streptomycin, isoniazid, or PAS were recovered from two of these, but their presence had no obvious untoward effect on the course of the illness.

The only patient who has not done well developed a tuberculous empyema as an immediate complication of artificial pneumothorax, carried out in the presence of a large tension cavity. In spite of this, he remained well for four years, with a chronic empyema, before his condition deteriorated. The remaining 10 patients have remained well and free of symptoms for periods from ten to fifty-one months after operation. In 5 of these there was a substantial drop in the insulin requirement after treatment; in the other 5 the dose has not greatly changed.

One patient, who had never had effective chemotherapy, had tubercle bacilli in the sputum on a single examination four years after thoracoplasty. The remaining 9 patients have shown no evidence of tuberculous activity, and in all 10 the diabetes remains stable.

There is no evidence in this small series that the diabetes, although severe in each case, had any very remarkable effect on the evolution of the tuberculosis; nor did it in itself cause any undue difficulty in the management of the patients.

SUMMARY

Eleven patients with cavitary tuberculosis complicating severe diabetes, and treated by major surgery, are described. The results are considered to justify an optimistic approach to this combination of diseases.

SUMARIO

El Tratamiento Quirúrgico de la Tuberculosis Pulmonar Que Complica la Diabetes Sacarina

Describense 11 casos en que enfermos que tenían cavernas tuberculosas que complicaban una diabetes grave fueron tratados con cirugía mayor. Considérase que los resultados justifican un aborde optimista a esta combinación de dolencias.

RESUME

Traitement chirurgical de la tuberculose pulmonaire compliquant un diabète sucré

Description de onze cas de tuberculose excavée compliquant un diabète sucré grave chez des malades qui furent traités par une intervention chirurgicale majeure. Les auteurs considèrent que les résultats permettent d'aborder avec optimisme le problème de l'association de ces affections.

Acknowledgments

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SEROLOGIC INVESTIGATIONS OF MYCOBACTERIAL ANTIGENS[1]

III. Direct Detection of Homologous Antibodies by BCG Extract Antigens in Serums of Rabbits Injected with BCG and Challenged with *Mycobacterium Bovis*

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INTRODUCTION

Investigations of the antibody response in naturally occurring or experimental tuberculosis have been impeded by the difficulties encountered in identifying species-specific antibodies.

An approach to the ready identification of mycobacterial antibodies was suggested when erythrocytes sensitized with antigens obtained by aqueous extraction of BCG (1) were found to react only with homologous antiserums. The present study was undertaken to determine if the specificity of the reaction could be applied to the direct identification of homologous antibodies in rabbits which had received more than one variety of mycobacteria.

PLAN OF INVESTIGATION

The experimental plan consisted of injecting rabbits with BCG and then challenging groups of these animals at intervals with *Mycobacterium tuberculosis* (var. *bovis*). This created different immunologic situations from which to determine if the antibodies detected with BCG extract-sensitized erythrocytes could be differentiated from those elicited by the superimposed infection.

Appropriate inhibition and adsorption tests were performed to confirm the specificity of the antibody measured by the extract-treated erythrocytes and to distinguish these antibodies from those demonstrated with OT-sensitized erythrocytes. The latter antigen preparation was employed since it will react with antibodies to many of the mycobacteria, including both BCG and *M. bovis*.

Materials and Methods

Schedule of injections: Nine young adult rabbits (each weighing approximately 2.0 kg.) were injected intravenously with 2.0 mg. of BCG, previously prepared as a homogeneous suspension in phosphate-buffered saline (pH 7.4). Subsequently, 8 of these animals were challenged intravenously with 0.02 mg. of virulent *M. bovis* (Ravenel), according to the schedule recorded in table 1. The ninth animal was not challenged and served as the BCG control. A tenth animal received *M. bovis* only.

Serums: Animals were bled at the intervals stated in table 2. All serums were recovered from blood samples obtained from the marginal ear veins and were clarified by centrifugation prior to being stored at −20°C.

Samples taken at the time of challenge were procured before the virulent inocula were introduced (table 2).

When all bleedings had been completed, aliquots of each of the stored serums were pre-

[1] From the Ohio Tuberculosis Hospital, Columbus, Ohio, and from the Department of Bacteriology, The Ohio State University, Columbus, Ohio.

[2] OT (4× International Standard) was kindly supplied by Dr. H. D. Piersma, Lederle Laboratories Division, American Cyanamid Corporation, Pearl River, New York.

TABLE 1

SCHEDULE OF CHALLENGE OF RABBITS IMMUNIZED WITH BCG (2.0 MG.)

Rabbit Number	Week of Challenge (0.02 mg. *M. bovis*)	Week of Final Bleeding Before Death
1 (BCG control)	—	—
2	7	17
3	7	20
4	5	17
5	5	17
6	3	17
7	3	11
8	1	17
9	1	9
10 (*M. bovis* control)	0	5

pared for serologic studies. The test serums were inactivated at 56°C. for thirty minutes and adsorbed twice at room temperature with 0.2 volumes of washed, packed sheep cells to remove naturally occurring antibodies. Although repeated freezing and thawing of samples was kept to a minimum, experience has shown this to have little or no effect on the antibody measurements under consideration.

Preparation of BCG extracts: Preparation of the aqueous extract was modified only slightly from the procedure described previously (1). After the supernatant fluids from the successive extractions had been filtered (Selas candles), a portion of each was dialyzed against deionized water for forty-eight hours. Aliquots of the first, third, sixth, ninth, and twelfth extracts, made isotonic by the addition of dry sodium chloride, were employed to sensitize sheep erythrocytes (*see* below).

Preliminary studies revealed that the sixth and ninth aqueous extracts were equally active and specific as erythrocyte-sensitizing antigens when tested with an homologous anti-BCG serum known to contain both hemolysins and hemagglutinins. Similar tests conducted with an heterologous anti-*M. bovis* serum failed to show evidence of cross-reactivity. Thus, it was unnecessary to concentrate the extracts to demonstrate specific antigenicity (1). Pools of equal portions of the sixth and ninth extracts, therefore, were employed as erythrocyte-sensitizing antigens in this investigation.

Sensitization of sheep erythrocytes with BCG extract and with OT: Four milliliters of isotonic BCG extract were mixed with 0.1 ml. of packed, washed sheep erythrocytes and incubated for two hours in a 37°C. water bath. The sensitized cells were thrice washed in buffered saline (pH 7.4) and employed in the serologic tests as a 0.5 per cent suspension.

The Old Tuberculin (OT)-sensitized cells were prepared by mixing 0.1 ml. of packed sheep cells with 6.0 ml. of a 1:15 buffered saline dilution of OT.[3] The incubation, washing, and final concentration of cells were the same as for the extract-treated erythrocytes.

Serologic tests: Eight different antibody tests were made with each test serum, four required BCG-sensitized erythrocytes as antigen, while the remaining four employed OT-treated erythrocytes. The details of the hemagglutination and hemolytic tests have been reported elsewhere (2) and consist essentially of "drop" modifications of standard procedures.

The heated-serum antibodies were demonstrated by applying the same tests to aliquots of adsorbed and inactivated serums, diluted 1:2 with saline and heated at 70°C. in a water bath for ten minutes (3).

RESULTS

Serologic patterns of immunized and challenged rabbits: Representative serologic patterns of the variously treated rabbits, as determined by the eight measurements just described, are recorded in table 2.

TABLE 2

ANTIBODY RESPONSE MEASURED WITH OT- AND BCG EXTRACT-SENSITIZED ERYTHROCYTES IN BCG-IMMUNIZED RABBITS CHALLENGED WITH M. BOVIS

Rabbit Number	Erythrocytes Sensitized with	Serologic Demonstration*	Weeks Following Immunization									
			0	1	3	5	7	9	11	13	15	17
1 (Control—BCG)	OT 1:15	HL	—	128	512	64	16	16	16	32	32	16
		HA	—	16	16	8	4	4	4	—	—	—
		ΔHL	—	—	—	4	4	4	4	2	2	4
		ΔHA	—	—	—	—	—	—	—	—	—	—
	BCG extract	HL	—	128	256	64	—	—	—	—	—	—
		HA	—	—	—	—	—	—	—	—	—	—
		ΔHL	—	—	—	16	—	—	—	—	—	—
		ΔHA	—	—	—	—	—	—	—	—	—	—
2	OT 1:15	HL	—	128	256	128	64	128	64	256	128	128
		HA	—	8	32	8	4	8	8	32	32	8
		ΔHL	—	—	—	8	—	16	32	64	128	64
		ΔHA	—	—	—	4	—	4	8	16	16	16
	BCG extract	HL	—	128	256	256	128	128	16	8	—	—
		HA	—	—	—	—	—	—	—	—	—	—
		ΔHL	—	—	—	32	32	16	8	—	—	—
		ΔHA	—	—	—	—	—	—	—	—	—	—
4	OT 1:15	HL	—	128	32	32	64	64	64	128	128	128
		HA	—	16	32	16	32	16	16	16	16	16
		ΔHL	—	—	—	—	—	4	16	32	32	32
		ΔHA	—	—	—	—	—	—	4	8	8	8
	BCG extract	HL	—	256	512	512	512	256	256	256	128	64
		HA	—	—	—	64	16	—	—	—	—	—
		ΔHL	—	—	—	4	32	8	4	4	4	—
		ΔHA	—	—	—	—	—	—	—	—	—	—
7	OT 1:15	HL	—	128	256	256	256	128	256	Died		
		HA	—	16	32	32	32	32	32			
		ΔHL	—	—	—	4	32	64	32			
		ΔHA	—	—	—	4	16	8	8			
	BCG extract	HL	—	128	1024	512	128	64	64			
		HA	—	—	—	—	—	—	—			
		ΔHL	—	—	—	8	64	32	16			
		ΔHA	—	—	—	—	—	—	—			
8	OT 1:15	HL	—	256	256	256	128	64	64	128	128	64
		HA	—	32	32	16	8	16	16	32	16	32
		ΔHL	—	—	4	8	16	32	64	32	32	32
		ΔHA	—	—	—	8	8	8	8	8	8	16
	BCG extract	HL	—	256	512	32	8	—	—	—	—	16
		HA	—	—	—	—	—	—	—	—	—	—
		ΔHL	—	—	16	16	8	4	—	—	—	—
		ΔHA	—	—	—	—	—	—	—	—	—	—
10 (Control—*M. bovis*)	OT 1:15	HL	—	256	512	256	Died					
		HA	—	16	128	16						
		ΔHL	—	—	4	8						
		ΔHA	—	—	4	8						
	BCG extract	HL	—	—	—	—						
		HA	—	—	—	—						
		ΔHL	—	—	—	—						
		ΔHA	—	—	—	—						

* HL and HA refer to hemolytic and hemagglutinin demonstrations, respectively; ΔHL and ΔHA refer to the same demonstrations, but with serums heated at 70°C. for ten minutes. All titers expressed as reciprocals of the serum dilutions. Results recorded at the left of heavy vertical lines indicate pre-challenge serums.

The serums from rabbit *No. 10* which had received only *M. bovis* failed to react with erythrocytes sensitized with BCG extract. This is consistent with the results of the preliminary experiments which were designed to select the extract(s) of desired specificity.

Of particular interest was the apparent absence of agglutinogen on the BCG extract-treated erythrocytes for the majority of serums tested; the serums from rabbit *No. 4* were the only exceptions. This same phenomenon was observed in the preliminary titrations with the standard serums which had demonstrated agglutinins with BCG extract-sensitized erythrocytes prepared with antigen from other lots of extract.

The unpredictable recovery of agglutinogen in the extractions from bacillary masses of BCG has been reported previously (1), but as yet no explanation for this has been established. The agglutinins detected in the serums from animal *No. 4*, therefore, must have been directed toward an antigen on the erythrocyte for which similar antibodies were lacking in the remainder of the test serums.

A comparison of the hemolytic patterns (unheated serums) as demonstrated with both OT and BCG antigen preparations strongly suggests that different antibodies were being measured with each cell preparation, although similar or even identical titers may have been observed with any one serum sample. In general, the titers resulting from testing serums from immunized and challenged animals with OT-sensitized erythrocytes exhibited little variation throughout the course of the experimentation, therefore differing from the more variable patterns seen with BCG-sensitized erythrocytes. The introduction of *M. bovis* into the BCG-immunized animals had little effect on the anti-BCG response, as shown by the titers and patterns seen with the extract-treated erythrocytes. In some animals, the anti-BCG hemolysins disappeared within a few weeks, while in others their disappearance was more gradual, and occasionally titers were seen to persist until death.

With OT-sensitized erythrocytes it was possible to detect some antibody in each serum sample from all animals. In serums from later bleedings, heat-stable hemolysins and hemagglutinins were uniformly observed, with the exception of serums from the BCG-control animal. The heated-serum antibodies, regardless of the test antigen, appeared later than did their labile counterparts, which confirms the observation of Dodd, Funkhouser, and Rheins (3).

Inhibition and cross-adsorption tests: To further establish the relationship of the antibodies measured with extract-treated cells and those detected with OT-sensitized erythrocytes, a series of inhibition tests was performed.

The procedure employed was a modification of the hemolytic titration, in which a drop of saline (control), OT (1:15), or BCG extract was added to each tube in the serum dilution series. After a preliminary incubation for thirty minutes at 37°C., two drops of the desired cell suspension and one drop of complement were added to each tube. Following reincubation, the hemolytic titer was determined. Abolition or reduction of titer when compared with the control test served to measure the inhibiting capacity of the test substance.

The serums were selected as representative of those obtained before and after

TABLE 3

INHIBITION OF HEMOLYSIS BY OLD TUBERCULIN AND BCG EXTRACT IN SERUMS FROM BCG-IMMUNIZED RABBITS BEFORE AND AFTER CHALLENGE WITH M. BOVIS

Erythrocytes sensitized with	Blocking Extract	Pre-Challenge							Post-Challenge											
		1-1*	1-5	4-1	4-3	4-5	7-3	4-7	4-7	4-9	4-11	4-13	4-17	7-5	7-7	7-11	4-3	4-5	10-17	10-3	10-5
OT, 1:15	Saline	128	64	128	64	32	128	64	64	128	128	256	128	128	128	128	256	128	256	512	256
BCG extract		32	64	64	256	256	256	128	256	128	128	256	128	64	64	64	256	32	—	—	—
OT, 1:15	OT, 1:15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BCG extract		4	32	32	256	256	256	64	256	128	128	128	128	32	64	16	128	8	—	—	—
OT, 1:15	BCG extract	16	4	4	8	16	16	16	64	32	32	64	64	16	32	64	64	32	64	512	128
BCG extract		8	16	16	64	64	64	16	64	64	64	32	32	16	16	16	32	16	—	—	—

* The first numeral refers to the rabbit number; the second refers to the week post immunization.
† Infected control—*M. bovis* only.

challenge with *M. bovis*. Serums from the control rabbit which had received *M. bovis* alone (animal *No. 10*) were included.

Although the results recorded in table 3 were those observed with unheated serums, similar relationships were evident from results of tests conducted with heated serums.

It can be seen that OT, 1:15, completely inhibited reactions between OT-sensitized erythrocytes and all antiserums. This has been reported previously (4). Old Tuberculin only infrequently affected reactions between BCG extract-treated cells and the test serums.

Titrations with both antigen preparations were inhibited only slightly by the addition of the BCG extract to the test systems. It is problematic whether or not the extract was more effective in preventing reactions between OT-sensitized erythrocytes and serums obtained before or after challenge.

To examine the possibility that the weak inhibiting property of the BCG extract was due to antigen deficiency, a sample was concentrated eight times by vacuum desiccation at room temperature, made isotonic, and re-tested with the same sensitized erythrocyte suspension and some of the serums used previously (table 4). As can be seen, the concentrated extract exhibited a markedly enhanced inhibiting capacity, particularly for pre-challenge serums, and emphasized the specificities not clearly evidenced in table 3. Since the extent of the concentration was chosen arbitrarily (eight times), there is little reason to believe that further treatment would not eliminate those anti-BCG demonstrations which persisted. Moreover, by concentrating the extract, the results of inhibition tests with post-challenge serums and OT-sensitized cells strongly suggest the presence of antibodies in those serums, with specificities directed only to antigens associated with the OT cells, and may be attributed to the effects of challenge with the virulent tubercle bacilli.

In the cross-adsorption tests, serum aliquots were completely adsorbed with either OT- or BCG extract-treated erythrocytes and were subsequently tested with the heterologous antigen preparation (table 5). The results indicated that

TABLE 4

INHIBITION OF HEMOLYSIS BY CONCENTRATED BCG EXTRACT

Erythrocytes Sensitized with	Blocking Extract	Hemolytic Titers															
		Pre-Challenge				Post-Challenge											
		4-3	4-5	7-3	8-1	4-7	4-9	4-11	4-13	4-17	7-5	7-7	7-11	8-3	8-5	10-1*	10-3	10-5
OT, 1:15	Saline	64	32	128	64	64	128	128	256	128	128	128	128	256	128	256	512	256
BCG extract		256	256	256	128	256	128	128	256	128	64	64	64	256	32	—	—	—
OT, 1:15	BCG extract, concentrated eight times	—	—	—	—	32	32	32	64	64	—	32	32	8	32	64	512	128
BCG extract		4	16	—	4	16	8	4	—	—	—	—	—	4	4	—	—	—

* Infected control—*M. bovis* only.

TABLE 5

TITRATIONS OF ANTISERUMS FOLLOWING ADSORPTION WITH OT- AND BCG EXTRACT-SENSITIZED ERYTHROCYTES

Treatment of Serums	Erythrocytes Sensitized with	Titer													
		Anti-BCG				Anti-*M. Bovis*				Immunized (BCG) and Challenged (*M. Bovis*)					
		2-5		5-3		10-1		10-3		5-7		9-5		5-9	
		H. L.*	H. A.	H. L.	H. A.	H. L.	H. A.	H. L.	H. A.	H. L.	H. A.	H. L.	H. A	H. L.	H. A.
Untreated	OT, 1:15	64	8	32	16	128	16	1024	128	32	4	128	16	32	—
	BCG extract	32	—	16	—	—	—	—	—	64	—	64	—	64	—
Adsorbed with 1:15 OT-sensitized erythrocytes	OT, 1:15	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	BCG extract	32	—	16	—	—	—	—	—	64	—	64	—	32	—
Adsorbed with BCG extract - treated erythrocytes	OT, 1:15	32	4	32	8	128	16	512	128	16	4	64	8	16	—
	BCG extract	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* HL and HA refer to hemolytic and hemagglutinin demonstrations, respectively.

adsorption with one of the antigen preparations did not significantly alter the antibody titer found with the other sensitized erythrocyte preparation. This was true whether the antiserums tested were obtained before or after challenge.

DISCUSSION

From the data presented it appears that erythrocytes sensitized with the appropriate BCG extract can be employed to detect directly specific antibodies in the serums of rabbits immunized with BCG and challenged with *M. bovis*. The independence of the serologic responses to immunization and to infection, as measured with OT- and BCG extract-treated erythrocytes, is shown by differ-

ences in antibody patterns and by the results of inhibition and cross-adsorption tests.

The effectiveness of the specific BCG (concentrated) extract in inhibiting reactions between OT-sensitized erythrocytes and test serums reflects the stage of immunization or challenge at which the serum samples were procured. Serums tested from animals prior to challenge were completely prevented from reacting with the OT-sensitized cells by the addition of BCG extract. After challenge, however, the extract was progressively less effective as inhibitor, which suggests the appearance of new antibodies with different specificities as the result of infection.

The results of the cross-adsorption studies are consistent with those of the inhibition tests in every respect but one. It might be anticipated from the results of the inhibition tests that the BCG extract-sensitized erythrocytes would adsorb all antibody from the pre-challenge serums capable of reacting with OT- and BCG-sensitized erythrocytes. However, this did not occur, suggesting that the extracts contain antigens which, although effective as inhibitors, failed to adsorb to the surface of erythrocytes or were not demonstrated in that capacity.

The presence in tuberculins of antigens with sufficient specificities to detect slight serologic differences among the mycobacteria has been recognized and investigated by other workers (5). However, the inhibition and adsorption procedures necessary to demonstrate these differences have not been readily applicable to the study of the changes which occur in the antibody response during immunization and infection. The BCG antigens employed in the present study not only identify specific homologous antibody, but also detect these substances in serums containing heterologous antibody as well.

Because of the possible value of applying similar procedures to clinical and other experimental studies, a more detailed description of the inhibiting and erythrocyte-sensitizing capacities of these antigens will be the subject of the following report.

SUMMARY

Erythrocyte-sensitizing antigens obtained by the aqueous extraction of bacillary masses of BCG can be used to detect directly homologous antibodies in rabbits immunized with BCG and challenged with a bovine strain of tubercle bacillus. The independence of the antibody measured with the BCG antigen from those detected with Old Tuberculin-sensitized erythrocytes was confirmed by inhibition and cross-adsorption tests.

SUMARIO

Investigaciones Serológicas de los Antígenos Micobacterianos: III. Descubrimiento Directo de Anticuerpos Homólogos por Antígenos de Extracto de BCG en Sueros de Conejos Inoculados con BCG y Provocados con Mycobacterium Bovis

Cabe usar antígenos eritrocito-sensibilizantes obtenidos por la extracción acuosa de masas bacilares de BCG para descubrir directamente anticuerpos homólogos en conejos inmunizados con una cepa bovina del bacilo tuberculoso. La independencia del anticuerpo medido con el antígeno de BCG de los descubiertos con eritrocitos sensibilizados con tuberculina antigua fué confirmada por pruebas de inhibición y de adsorción cruzada.

RESUME

Investigations sérologiques des antigènes mycobactériens: III. Détection directe d'anticorps homologues par les antigènes d'extraits de BCG dans le sérum de lapins inoculés avec du BCG et soumis à l'action du Mycobactérium bovis

Des antigènes sensibilisateurs de l'érythrocyte, obtenus à partir de masses bacillaires de BCG par extraction aqueuse, peuvent être utilisés pour déceler directement les anticorps homologues chez des lapins immunisés avec le BCG et soumis à l'action de la souche bovine du bacille tuberculeux. L'indépendance de l'anticorps déterminée par l'antigène du BCG par rapport à ceux décelés avec des érythrocytes sensibilisés par la tuberculine de Koch, fut confirmée par les tests d'inhibition et d'adsorption croisée.

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SEROLOGIC INVESTIGATIONS OF MYCOBACTERIAL ANTIGENS[1, 2]

IV. Further Serologic Characterization of BCG Extract Antigens

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INTRODUCTION

Studies concerned with the preparation and serologic specificity of aqueous extracts of BCG have been the subject of the preceding report (1) and other recent publications from these laboratories (2, 3). The apparent effectiveness of sheep erythrocytes sensitized with these extracts in identifying the homologous antibodies in serums containing heterologous substances as well suggests a level of antigenic specificity not often encountered with components recovered from mycobacteria.

Fundamental to successful demonstrations of antigen-antibody reactions by indirect hemagglutination or hemolysis is the adsorption of antigen to the erythrocyte carrier. The term *indirect* is employed to designate those procedures in which antigens are demonstrated after their adsorption to erythrocyte carriers and to distinguish such antigens from the iso-antigens. Landy (4) has shown that normal erythrocytes exhibit a maximal adsorptive capacity for a given antigen, although several different antigens may be adsorbed to the same erythrocyte. Chemical treatment of the erythrocyte, as with tannic acid (5), may modify its surface so as to create new or additional "receptor sites" and thus permit the adsorption of antigens not normally removed by the untreated erythrocyte. By the use of such procedures, in conjunction with proper antiserums, it has been possible to detect minor differences among closely related antigens (6).

Another technique of value in antigenic analyses is that of gel precipitin reactions which measure the independent diffusion in gels of antigens in a mixture.

The following series of experiments was undertaken, employing the above techniques and their modifications, to characterize further serologically the BCG extracts. For purposes of comparison, Old Tuberculin (OT) was subjected to the same testing procedures whenever feasible.

MATERIALS AND METHODS

BCG-aqueous extracts and sensitization of erythrocytes: The extracts employed in this study were prepared as were those described previously (2, 3).

The erythrocyte-sensitizing procedures and methods of performing the serologic tests (hemolytic and hemagglutination) also have been described (7).

In the present series of experiments, both sheep and human erythrocytes were tested

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[1] From the Department of Bacteriology, The Ohio State University, and from the Ohio Tuberculosis Hospital, Columbus, Ohio.

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as antigen carriers. The latter erythrocytes were identified as belonging to *Group O*, and were Rh positive.

Tanned erythrocytes (8): One-tenth milliliter of washed, packed sheep erythrocytes was mixed with 1.0 ml. of a 1:10,000 saline dilution of tannic acid (Mallinckrodt, fluffy). The mixture was incubated for ten minutes in a 37°C. water bath, after which the tanned cells were recovered by centrifugation and washed three times with saline. These tanned erythrocytes were sensitized with BCG extract or OT according to the usual procedure.

Serums: The test serums were those used in the preceding study (1), but with one exception. An hyperimmune anti-BCG serum was obtained from a rabbit which had received multiple intravenous injections of viable BCG (2.0 mg.). This serum was utilized as one of the reactants in the agar-diffusion tests (*see* below).

Agar-diffusion (9): Filter paper discs (12.7 mm.) were saturated with either antiserum or antigen (BCG extract, OT, 1:30) and dried *in vacuo*. By repeating this process twice, an effective concentration of reactants was accomplished. The discs were placed 7.0 mm. apart on 0.8 per cent agar plates. The agar, prepared with saline, contained 1:10,000 merthiolate and 1.0 M glycine (10) to minimize nonspecific precipitation. The pH had been adjusted to 7.6. The reactants again were applied to the discs (threefold concentration) with dropper pipettes and the plates were incubated at 4°C. for fourteen days. Observations were made every two days by viewing the plates in oblique light. The time of appearance and number of lines of precipitate were noted.

RESULTS

Adsorption of BCG extract and OT to sheep erythrocytes: As one aspect of studying the serologic characteristics of the BCG extract antigens, an attempt was made to determine if the sites on the sheep erythrocyte at which these antigens are adsorbed are the same as those required by OT.

The erythrocyte-sensitizing procedure employed was only slightly modified from that described by Landy (4) to demonstrate the independence of erythrocyte adsorption of different antigens. One-tenth milliliter quantities of washed, packed normal sheep erythrocytes were sensitized with either OT, 1:15, or BCG extract, a mixture of these antigens, or by successive addition of the antigens. All antigen-erythrocyte mixtures were incubated for a total of four hours in a 37°C. water bath, i.e., erythrocytes which were successively treated were incubated for two hours with each antigen; the other preparations were incubated for the four-hour period. Preliminary tests failed to show any adverse effects because of additional incubation.

The sensitized erythrocytes were thrice washed with phosphate-buffered saline and were employed as 0.5 per cent saline suspensions.

An anti-BCG serum and an anti-*M. bovis* serum of comparable titers, when tested with OT-sensitized erythrocytes, were titrated for hemolysins and hemagglutinins with each of the above antigen preparations (table 1).

The reactions of both serums with the BCG extract-treated erythrocytes were as anticipated; the anti-*M. bovis* serum failed to react, while the anti-BCG serum produced hemolysis only (1). The treatment of erythrocytes with BCG extract in conjunction with OT, regardless of the sequence of treatment, resulted in titers identical to those seen with OT-sensitized erythrocytes. Thus, it was tentatively assumed that the erythrocyte-sensitizing antigens present in OT and in the BCG extract were adsorbed to different sites.

TABLE 1

SIMULTANEOUS AND SUCCESSIVE ADSORPTION OF BCG EXTRACT AND OT TO SHEEP ERYTHROCYTES

Sheep Erythrocytes Sensitized with	Test Serums			
	Anti-BCG		Anti-*M. bovis*	
	H.L.*	H.A.	H.L.	H.A.
OT, 1:15	128	16	128	16
BCG extract	32	—	—	—
Mixture of OT, 1:15, and BCG extract	128	16	128	16
OT, 1:15, followed by BCG extract	128	16	128	16
BCG extract followed by OT, 1:15	128	16	128	16

* H.L. refers to hemolysis; H.A. to hemagglutination. Titers are expressed as reciprocals of serum dilutions.

TABLE 2

ADSORPTION OF ANTISERUM WITH VARIOUSLY SENSITIZED ERYTHROCYTES

Sheep Erythrocytes Sensitized with	Reaction	Treatment of Anti-BCG Serum			
		I	II	III	IV
		Untreated	Adsorbed with OT (1:15)-Sensitized Erythrocytes	Adsorbed with BCG Extract-Sensitized Erythrocytes	Adsorbed with Erythrocytes Sensitized with Mixture of OT, 1:15, and BCG Extract
OT, 1:15	H.L.*	128	—	128	—
	H.A.	16	—	16	—
BCG extract	H.L.	32	32	—	—
	H.A.	—	—	—	—
Mixture of OT, 1:15, and BCG extract	H.L.	128	32	128	—
	H.A.	16	—	16	—

* H.L. refers to hemolysis; H.A. to hemagglutination.

☐ indicates homologous adsorption.

Cross-adsorption tests: In view of these results, aliquots of the same anti-BCG serum were adsorbed completely at room temperature with either OT(1:15)-sensitized erythrocytes, erythrocytes treated with the BCG extract, or a mixture of OT and BCG extract. Following the adsorptions, each serum sample was tested with the homologous and heterologous antigens.

As shown in column IV of table 2, adsorption of the test serum with erythrocytes sensitized by the mixture of OT, 1:15, and BCG extract removed all antibodies. The selective removal of only homologous antibodies by the other adsorptive procedures (*see* columns II and III of table 2) confirms the independence of the antibodies measured by the two antigen preparations since heterologous titers were unaffected. Furthermore, since these data show that both antigens

are adsorbed simultaneously to the erythrocyte surface, it is suggested that the antigens in OT and the BCG extract are not adsorbed to the same loci on the sheep erythrocyte.

Sensitization of tanned erythrocytes by BCG extract and OT: The work of Boyden (5) and others (11) has shown that tanning of erythrocytes permits the adsorption of proteins, a phenomenon not usually attributed to untreated erythrocytes. However, both untreated and tanned erythrocytes may adsorb certain carbohydrates.

It seemed advisable, therefore, to determine if the BCG extract contained antigens which might be demonstrated with the chemically altered erythrocytes. Accordingly, washed, packed sheep erythrocytes were tanned by the procedure described above. One-tenth milliliter of tanned erythrocytes then was sensitized with 4.0 ml. of BCG extract by the usual method. Another portion of the extract was adsorbed with untreated erythrocytes to remove the readily adsorbable antigenic substances prior to the addition of tanned erythrocytes. Similar procedures were carried out with OT, 1:15. BCG extract and OT-sensitized normal erythrocyte suspensions were prepared as control antigens. Twenty serums were examined for hemolysis and hemagglutination with the various treated erythrocyte suspensions. Only qualitative tests were performed.

The tanning of erythrocytes seemingly had no effect on the sensitizing properties

TABLE 3

SENSITIZATION OF NORMAL AND TANNED ERYTHROCYTES WITH UNTREATED AND ADSORBED BCG EXTRACT AND OT

Test Antigens	Reaction	Serums																				
		Anti BCG					Anti-*M. Bovis*				Anti-*M. Hominis*		Anti *M. Phlei*				Serums from Rabbits Immunized (BCG) and Challenged (*M. Bovis*)					
		1	2	3	4	5	1	2	3	4	1	2	1	2	3	1	2	3	4	5	6
BCG extract-sensitized erythrocytes	H.L.	+	+	+	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
	H.A.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BCG extract-sensitized tanned erythrocytes	H.L.	+	+	+	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
	H.A.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BCG extract-(adsorbed) tanned erythrocytes	H.L.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	H.A.	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–
OT (1:15)-sensitized erythrocytes	H.L.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	H.A.	+	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
OT (1:15)-sensitized erythrocytes	H.L.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	H.A.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
OT (1:15)-(adsorbed) tanned erythrocytes	H.L.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	H.A.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

or specificity of the unadsorbed BCG extract (table 3). The test serums, including those from animals which had been immunized and challenged, reacted identically with both the extract-sensitized tanned erythrocyte and normal erythrocyte antigens. Similar results were not found, however, when serums were tested with tanned and normal erythrocytes sensitized with unadsorbed OT. The absence of agglutinin demonstrations with OT-sensitized tanned erythrocytes has been observed previously (3). The failure of the serums to react with tanned erythrocytes treated with the adsorbed OT may be explained by the complete adsorption of the lytic antigens by the normal erythrocytes.

The erratic agglutination seen with tanned erythrocytes sensitized with the BCG extract, previously adsorbed with normal erythrocytes, is unexplained although it was considered possible that it may have represented naturally occurring antibody in the serums of some rabbits for a modification induced by this particular erythrocyte treatment.

Heat stability of antigens in BCG extract: Aliquots of the BCG extract were heated for ten minutes at the following temperatures: 50°C., 65°C., 80°C., and 100°C. Subsequently each heated extract sample was used to sensitize normal erythrocytes, and the resulting antigens were tested by the usual methods with an anti-BCG serum.

None of the heat treatments employed affected the serologic demonstrations, since the test serum reacted to the same titers with all antigen preparations.

BCG extract sensitization of human erythrocytes: The use of human erythrocytes as the antigen carrier of OT (12) led to a similar study with the BCG extract under consideration.

Human *Group O* (Rh-positive) erythrocytes were treated with BCG extract according to the usual procedure. Complement and the standard anti-BCG serum were adsorbed twice at room temperature (fifteen minutes per adsorption) with equal quantities of washed, packed, human erythrocytes before testing.

As may be seen in table 4, the treatment of human erythrocytes with the extract failed to reveal the presence of antigens on the erythrocytes, as evidenced by the lack of demonstration with the anti-BCG serum. However, adsorption of the serum with the extract-treated human erythrocytes removed the hemolytic antibodies as shown by re-testing the adsorbed serum with extract-sensitized sheep erythrocytes.

TABLE 4

BCG Extract Sensitization of Human Erythrocytes

Test Antigen	Titer of Anti-BCG Serum			
	Before Adsorption with Extract-Treated Human Erythrocytes		*After* Adsorption with Extract-Treated Human Erythrocytes	
	H.L.*	H.A.	H.L.	H.A.
BCG extract-treated sheep erythrocytes	128	—	—	—
BCG extract-treated human erythrocytes	—	—		

* H.L. refers to hemolysis; H.A. to hemagglutination.

Greater concentrations of complement than 1:9 were introduced into a system containing eight antibody units to determine if lysis could be incurred. The only reaction observed was in the test containing undiluted complement, and in this instance only partial lysis resulted.

Agar-diffusion studies: Previous studies from these laboratories (9, 13) have shown the applicability of double-diffusion techniques, employing gel menstruums, to the study of tuberculins and antimycobacterial serums. The experiments described thus far were concerned only with the erythrocyte-sensitizing properties of the BCG extract, and it was believed that additional information regarding the antigenic components of the extract might be gained from diffusion studies. For purposes of comparison, OT and several BCG extracts, in addition to the extract employed for sensitizing erythrocytes, were tested with the anti-BCG serum.

After varying the concentrations of the reactants to obtain maximal demonstrations, it was observed that each of the test extracts elicited two lines of precipitate with the particular anti-BCG serum employed. Old Tuberculin often gave three to four distinct precipitates. The time required for the appearance of the precipitates differed with the concentrations of antigens in the extract being examined and was probably due to the time necessary for the diffusing reactants to reach the equivalent proportions at which the reactions became visible. Adsorption of the extracts and OT with normal sheep erythrocytes prior to the agar-diffusion tests did not alter their reactivity. The results of preliminary tests to determine if prior adsorption of the extracts with tanned erythrocytes would alter their antigenicity indicated that the antigens responsible for the reactions were not removed by the adsorption procedure. However, similar adsorption of OT resulted in lessened reactivity, since fewer lines of precipitate were observed.

DISCUSSION

The complexities of the erythrocyte surface structures have been well exemplified by the results of many serologic investigations. It is well recognized, for instance, that influenza virus will hemagglutinate erythrocytes from only certain animal species. Furthermore the agglutination of erythrocytes by a particular virus does not necessarily preclude agglutination of the same erythrocytes by other viruses (14). Chemical modification (15, 16), as well as virus treatment of erythrocytes (17), often results in sufficient alteration of the erythrocyte to confer new or additional antigenic properties. The evidence presented by Landy (4) for the simultaneous adsorption of bacterial antigens derived from several genera is confirmed by the present findings and has been extended to include antigens from closely related species (*M. tuberculosis* var. *hominis* and BCG). Although OT undoubtedly contains a mixture of antigens, several of which are adsorbable to the sheep erythrocyte, their presence did not interfere with the adsorption of antigens from the BCG extract.

The tanning of erythrocytes as a means of favoring the adsorption of proteins had no effect on the demonstration of antigens in the BCG extract. This, however,

should not be interpreted as meaning that the specificity of the extract is attributed only to carbohydrate. It is conceivable, as postulated previously (3), that carbohydrate-protein complexes may be adsorbed to the erythrocyte surface, with the protein portion of the complex directing the specificity. The removal of antigens by normal erythrocytes left little, if any, antigenic material to be adsorbed by the tanned erythrocytes.

The erythrocyte-sensitizing capacity of the BCG extract seemingly was not modified by the temperatures and time of treatment investigated in these studies. No attempt was made, however, to relate the extract antigens to the α hemosensitin of Sorkin and Boyden (18). Whether or not heating of the bacillary mass (BCG) prior to aqueous extraction would affect the antigenic specificity of the extracts remains to be seen. This would be of particular interest since the preparation of OT is accomplished by prolonged heating at elevated temperatures. This treatment may be partly responsible for the lack of specificity found with OT-sensitized erythrocytes, or perhaps OT is analogous to the first BCG extracts. Erythrocytes sensitized with early BCG extracts reacted with antiserums to several of the mycobacteria.

Human erythrocytes can be sensitized with BCG extract antigens despite the failure to demonstrate the sensitization with the serologic methods employed (hemolysis or hemagglutination). Adsorption of anti-BCG serum with these sensitized erythrocytes removed antibodies, as was shown by testing the supernatant fluids. Since, in the writers' experience, OT-sensitized human erythrocytes did not lyse in the presence of guinea pig complement, although agglutination was observed, complement from other animal species possibly would have overcome this difficulty. Other techniques such as the conglutinin-complement-adsorption test may aid in explaining these phenomena.

The results of another method for antigen analysis, the agar-diffusion reaction, suggest that the BCG extracts, as well as OT, contain antigens other than those adsorbable to erythrocytes. This conclusion was also reached by Boyden and Sorkin (19), who observed that certain of their mycobacterial fractions, although inactive by hemagglutination tests, gave precipitates with antiserum in agar.

The choice of test serum for these demonstrations markedly influences the results. A given serum may contain antibodies to minor antigens and thus demonstrate those antigens in the diffusion reaction. Another serum, lacking the same antibodies, would lead to false conclusions concerning the antigen content of the fraction being examined. An hyperimmune anti-BCG serum was chosen deliberately for these reasons.

SUMMARY

The antigens present in aqueous extracts of BCG and in Old Tuberculin are adsorbed to different sites on the sheep erythrocyte as shown by cross-adsorption tests.

The sensitization of tanned erythrocytes with BCG extract did not demonstrate antigens other than those observed with sensitized normal erythrocytes. The erythrocyte-sensitizing capacity of the antigens in BCG extract was unaffected

by temperatures up to and including 100°C. Although human erythrocytes adsorbed antigens from BCG extract, the addition of known reactive serums and guinea pig complement did not result in hemolysis. BCG extract also contains antigens capable of precipitating antibodies in the agar-diffusion tests. Previous adsorption of the extract with normal or tanned erythrocytes did not influence this reactivity with the particular test serum employed.

SUMARIO

Investigaciones Serológicas de los Antigenos Micobacterianos: IV. Nueva Caracterización Serológica de los Antigenos de Extractos de BCG

Los antigenos presentes en extractos acuosos de BCG y en la tuberculina antigua se adsorben en distintos sitios de los eritrocitos de oveja según revelan pruebas de adsorción cruzada.

La sensibilización de eritrocitos curtidos con extracto de BCG no reveló antigenos distintos de los observados en eritrocitos normales sensibilizados. La capacidad eritrocitosensibilizante de los antigenos de extracto de BCG no fué afectada por temperaturas hasta de 100°C. inclusive. Aunque los eritrocitos humanos adsorbieron antigenos del extracto de BCG, la adición de sueros reactivos conocidos y de complemento de cobayo no culminó en hemólisis. El extracto de BCG contiene además antigenos capaces de precipitar anticuerpos en las pruebas de difusión en agar. La adsorción previa del extracto con eritrocitos curtidos o normales no afectó esta reactividad con el suero empleado en la prueba.

RESUME

Investigations sérologiques des antigènes mycobactériens: IV. Nouvelle détermination des caractères des antigènes d'extraits de BCG

Les antigènes présents dans les extraits aqueux de BCG et dans la tuberculine de Koch sont adsorbés en différents points sur l'érythrocyte de mouton, comme le démontrent les tests d'adsorption croisée.

La sensibilisation d'érythrocytes tannés avec l'extrait de BCG n'a pas révélé d'autres antigènes que ceux observés avec les érythrocytes normaux sensibilisés. Le pouvoir de sensibilisation de l'érythrocyte des antigènes d'extraits de BCG n'était pas affecté par les températures atteignant 100°C. inclusivement. Malgré l'adsorption des antigènes de l'extrait de BCG par les érythrocytes humains, l'adjonction de "réactive sérums" déterminés et de complément de cobaye n'entralnait pas l'hémolyse. L'extrait de BCG contient également des antigènes capables de précipiter les anticorps dans les tests de diffusion sur gélose. Avec le sérum particulier utilisé dans ce test, l'adsorption antérieure de l'extrait avec les érythrocytes normaux ou tannés n'a pas influencé cette réactivité.

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Case Reports

STEATORRHEA AND PROBABLE TUBERCULOSIS WITH ACQUIRED HYPOGAMMAGLOBULINEMIA[1,2]

H. H. ZINNEMAN AND WENDELL H. HALL

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Tuberculosis has been infrequent in adults with acquired hypogammaglobulinemia and in children with the congenital form of the disease. Steatorrhea has been described in five cases of acquired hypogammaglobulinemia. The patient to be reported had steatorrhea and probable tuberculous mesenteric lymphadenitis before the onset of hypogammaglobulinemia. The data on this patient shed some light on the pathogenesis and immunologic aspects of this curious syndrome.

CASE REPORT

K. L. N., a service station operator, born in 1920, had measles, chicken pox, mumps, and whooping cough during childhood, without complications. He also had one episode of pneumonia in 1938. In 1941, shortly after his induction into the army, he had an episode of jaundice. In 1942, while in India, he developed diarrhea typical of "tropical sprue." Since 1943 he has had attacks of cough, fever, and fatigue with increasing frequency. In 1946 he had severe pneumonia followed by anorexia, chronic cough, occasional bouts of fever, and weight loss. The steatorrhea continued. A chest roentgenogram in 1947 showed bilateral apical pulmonary fibrosis with calcification in the left hilus, suggesting tuberculosis. Nineteen sputum cultures and several cultures of aspirated gastric contents were negative for tubercle bacilli. Sputum cultures for various fungi were negative. Gross steatorrhea persisted, and a roentgenographic evaluation of the gastrointestinal tract presented a "deficiency pattern." A megaloblastic anemia was controlled successfully with folic acid and vitamin B_{12}. A skin test with 0.005 mg. of PPD resulted in a 2-plus reaction.

In September, 1947, the subject had pneumonia involving the apical portion of the right lung and the left midlung field. In June, 1948, he was treated for pneumonia of the left lower lobe. Measurements of his serum proteins by a modification of the Kingsley method were 5.0 gm. per 100 ml. with 3.8 gm. albumin and 1.2 gm. globulins.

In February, 1949, an exploratory laparotomy was performed because of epigastric pain; roentgenographic and gastroscopic changes in the gastric mucosa suggested the possibility of a scirrhous carcinoma. Upon surgical exploration the serosal surfaces of the stomach and intestines presented multiple small grayish-white nodules, 1 to 2 mm. in diameter. A subtotal gastric resection was performed. Histologic examination showed a purulent gastritis with destruction of the mucosal pattern. There were no plasma cells. The serosa contained miliary tubercles (figure 1). One mesenteric lymph node presented caseous necrosis surrounded by lymphocytes, epithelioid cells, and giant cells of the Langhans' type (figure 2). Several other lymph nodes revealed only reactive hyperplasia. Plasma cells were absent. The surgical material was not cultured. Ziehl-Neelsen and periodic acid-Schiff stains of the histologic sections failed to reveal acid-fast organisms or yeast cells. Streptomycin was administered to the patient for only five days after the operation. Rapid recovery followed, without clinical signs of peritonitis.

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[2] Presented before the Medical Session, as part of *Section 2B*, at the annual meeting of the National Tuberculosis Association, New York, New York, May 21, 1956.

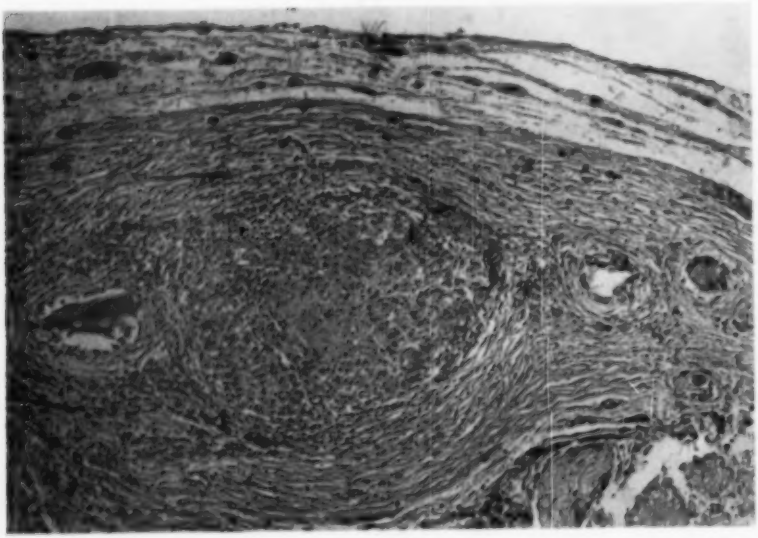

FIG. 1. Tubercle at the serosal surface of the resected stomach. There are epithelioid cells, giant cells, and lymphocytes with a fibrous wall but little central necrosis. Magnification × 75.

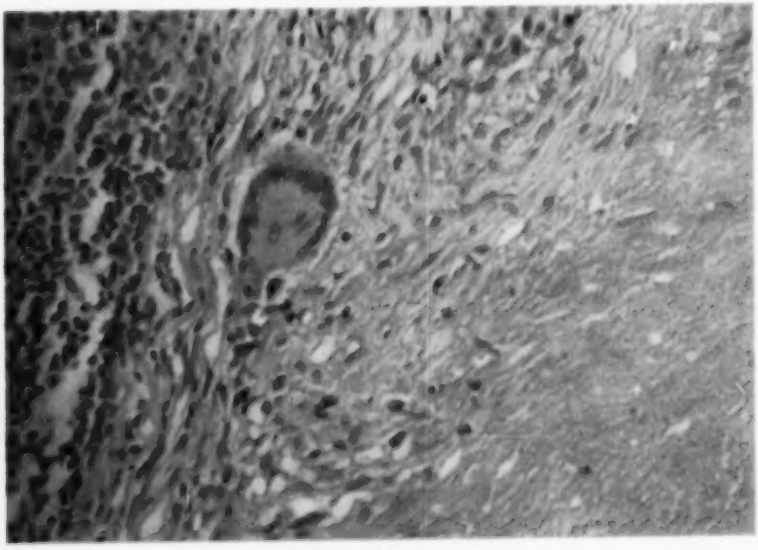

FIG. 2. Caseous necrosis in a mesenteric lymph node. A giant cell is seen amid epithelioid cells. Part of the necrotic area is seen to the right. Magnification × 200.

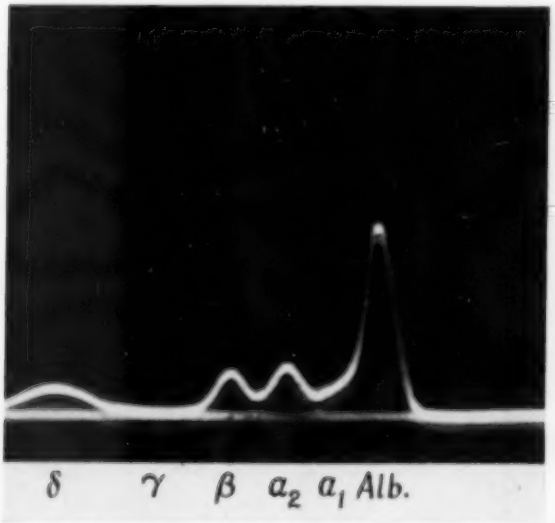

FIG. 3. Electrophoretic pattern of the patient's serum shows the apparent absence of gamma globulin by the Tiselius method.

In March, 1949, the patient was treated for pneumonia of the left upper lobe, which again responded well to penicillin. Total serum proteins at that time were 5.9 gm. per 100 ml., with 5.1 gm. albumin and 0.8 gm. globulins. These fractions were essentially the same on four repeated determinations. Chemical fractionation of the serum proteins by Jager's modification of the Weichselbaum salt precipitation method resulted in the following values: albumin, 4.1 gm. per 100 ml.; alpha globulins, 1.2 gm.; beta globulin, 0.4 gm.; and gamma globulin, 0.1 gm. Other laboratory examinations revealed normal urine findings and leukocytes ranging from 4,600 to 14,700. The erythrocyte sedimentation rate usually was 1 or 2 mm. in one hour, but rose to 27 mm. on one occasion.

A connection between the low gamma globulin level and the multiple infections was postulated at that time (1949) by the resident physician. The patient continued to be subject to frequent episodes of pneumonia, all of which cleared satisfactorily on penicillin therapy.

In 1954, on the occasion of another pneumonic infiltrate of the left upper lobe of the lung, this patient's immunologic status was studied. At that time the entity of hypogammaglobulinemia had become established and 3 patients with acquired hypogammaglobulinemia had been observed by the writers within the preceding ten months (1). Electrophoretic analysis of the serum proteins by the Tiselius method resulted in the following values (also figure 3): albumin, 3.4 gm. per 100 ml.; alpha$_1$ globulin, 0.5 gm.; alpha$_2$ globulin, 1.0 gm.; beta globulin, 0.8 gm. There was no gamma globulin. Immunochemical determination of gamma globulin[2] revealed the presence of 96.5 mg. per 100 ml. of serum. The serum zinc sulfate turbidity was zero. Skin tests with histoplasmin and

[2] Determination in the laboratories of Dr. R. A. Good, Minneapolis, Minnesota.

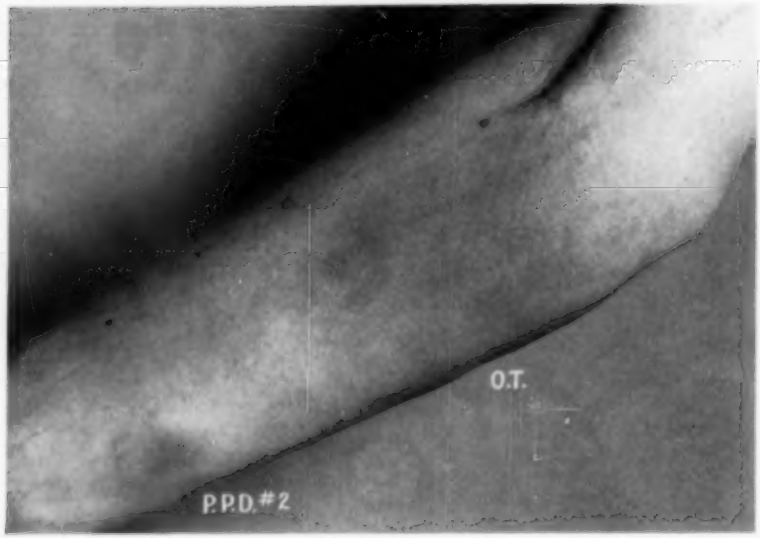

FIG. 4. Skin tests with Old Tuberculin (OT) and 0.005 mg. of purified protein derivative (PPD #2). Note the lymphangitis arising from the OT skin reaction.

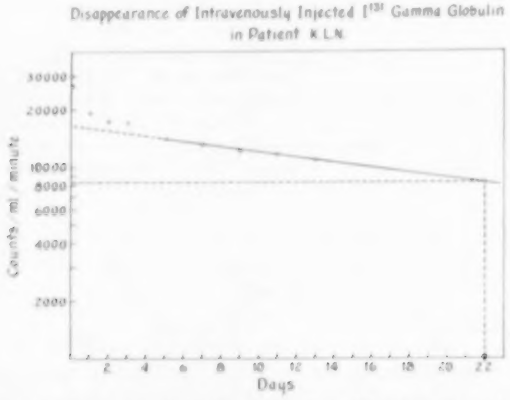

FIG. 5. Counts per minute are plotted on a semilogarithmic scale against time. The disappearance of one-half the intravenously injected I^{131}-labeled human gamma globulin is delayed to twenty-two days or about twice the normal time.

coccidioidin antigens in dilutions of 1:1,000 were negative. Skin tests with 0.00002 mg. and 0.0002 mg. of PPD gave positive reactions. A skin test with OT also was strongly positive (figure 4). Challenges by subcutaneous injection of 0.08 mg. of the polysaccharide of *Diplococcus pneumoniae*, type I, and two successive doses of 0.5 ml. of typhoid-paratyphoid vaccine were not followed by

antibody formation demonstrable by tests for precipitins and agglutinins. The Schick test remained positive after repeated injections of the antigen.

The disappearance rate of intravenously injected I^{131}-labeled human gamma globulin was calculated at a half time of twenty-two days (figure 5). The half time in normal subjects was found to be 10.5 days plus-minus 1.5 days. The exchangeable pool of gamma globulin was calculated at 2.5 gm.[4] (2). The patient's erythrocytes contained antigens of type *A* and were Rh-negative. His serum failed to agglutinate Rh-positive cells of *Group B* before and eight days after intravenous injection of 1.0 ml. of whole blood from an Rh-positive donor of *Group B*. Examination of material obtained by biopsy of the sternal bone marrow revealed no plasma cells.

Since January, 1955, the patient has been given prophylactic therapy with 400,000 units of penicillin orally per day in addition to the medical management of his steatorrhea. He has remained free of infections since that time and is engaged in gainful employment.

COMMENT

Several aspects of this patient's disease go beyond the usual problem of recurrent pyogenic infections associated with hypogammaglobulinemia. The granulomatous disease which involved his gastrointestinal tract may have been the cause of his steatorrhea. The hypogammaglobulinemia seemed to develop gradually after the appearance of steatorrhea and pulmonary fibrosis. Bacteriologic confirmation is missing, but the gross appearance of the peritoneum at the time of laparotomy, the histologic findings, the apical pulmonary fibrosis, and the positive reactions to PPD and OT strongly suggest tuberculosis.

The presence of tuberculous hypersensitivity in this patient is most interesting. It has been maintained by some that positive Mantoux reactions could not be expected in hypogammaglobulinemia, since these patients lack the classical anaphylactic antibodies contained in the gamma globulin fraction of the serum. The immediate type of skin reaction, as observed in the Arthus phenomenon, indeed is produced by these antibodies. The delayed type of bacterial hypersensitivity, however, is the result of a different mechanism. Tuberculin hypersensitivity can be induced in the guinea pig by the combination of a protein and a wax of the tubercle bacillus (3–6). The tuberculoprotein provides the antigenic stimulus, while the wax appears to influence the nature of the animal's hypersensitive response. Such an experimental hypersensitivity to tuberculoprotein is not necessarily associated with an increase in resistance to infection with tubercle bacilli (4). On the other hand, repeated injections of tuberculin may abolish hypersensitivity in an infected animal without influence on its acquired resistance (7, 8). The tuberculoprotein antibodies responsible for the delayed type of hypersensitivity to tuberculin have been believed to be cell bound. Passive transfer of tuberculin sensitivity cannot be accomplished with the whole serum of a sensitized (tuberculin-positive) animal, but the transfer

[4] The value in normal adults is approximately 50 gm.

of its intact or disrupted cells will convey local tuberculin sensitivity to a normal animal (6).

Cole and Favour (9) recently isolated antibodies to tuberculoprotein in the serum of sensitized guinea pigs. These antibodies were found in a subfraction (IV-10) of the alpha globulins, which represented 0.6 per cent of the total plasma proteins. With this fraction they were able to passively transfer the delayed type of dermal reaction to PPD in guinea pigs. The gamma globulin fraction completely inhibited the delayed type of skin reaction to fraction IV-10. It remains to be seen whether fraction IV-10 is a primary plasma component or whether it was originally contained in leukocytes and then liberated into the plasma by the disintegration of these cells. In any event, the work of Cole and Favour is of fundamental importance. It confirms the observation that the antibodies producing hypersensitivity of the delayed type to bacteria are not located in the gamma globulin fraction. It would have been highly desirable to attempt the passive transfer of tuberculin hypersensitivity with the serum of the patient, since alpha globulins were present and gamma globulin was absent. However, such an experiment was not deemed advisable because he had had hepatitis in the past.

In the light of all this, it is to be expected that the occurrence of hypogammaglobulinemia should not abolish the tuberculin reaction. The present literature concerned with acquired hypogammaglobulinemia contains the description of three additional patients with positive tuberculin reactions (10–12). A recent report (13) demonstrated the successful passive transfer of tuberculin hypersensitivity to a patient with acquired hypogammaglobulinemia by means of injection of intact leukocytes from a tuberculous donor. Two patients with congenital hypogammaglobulinemia developed positive tuberculin tests following BCG vaccination (14).

The resistance of the patient in this report against what appears to have been tuberculous mesenteric lymphadenitis and peritonitis seems remarkable. Existing tuberculosis in patients who have acquired hypogammaglobulinemia has been benign and easily managed (11). There has been one case of primary pulmonary tuberculosis with tubercle bacilli in the gastric aspirates in a patient with congenital hypogammaglobulinemia. The clinical course was benign. Inoculation of subjects with hypogammaglobulinemia with live BCG vaccine has not been followed by spreading infection (14).

Patients with hypogammaglobulinemia and demonstrable lack of antibodies are an easy prey to infections with pneumococci, meningococci, and streptococci, but do not appear to be defenseless against a tuberculous infection. Serum antibodies to tubercle bacilli have been difficult to demonstrate regularly in patients with tuberculosis. It is likely that resistance to tuberculous infection is the result of intracellular destruction of tubercle bacilli by phagocytes under the influence of metabolic and hormonal factors.

The hypogammaglobulinemia in the patient in this report may have been the result of his primary granulomatous disease (tuberculosis). An involvement of the lymphatic system occurs with acquired hypogammaglobulinemia with

striking frequency. Among thirty-two patients with acquired hypogammaglobulinemia (1, 10–13, 15–31), notable change in the lymphatic system was found in eighteen. In seven of them (1, 11, 13, 15, 21, 26, 31), some lymphatic disease clearly antedated the development of hypogammaglobulinemia. Lymphatic hyperplasia was noted in four instances (12, 22, 24), histoplasmosis in one (21), tuberculosis in two (11), probable tuberculosis in the case under discussion and in an additional one described by Rohn (19). Hodgkin's disease and chronic lymphatic leukemia were evident in four cases (12, 30), Boeck's sarcoid in two (1, 15, 28), and thymoma in three (13, 26, 31).

Including the patient in this report, six and probably seven of a total of thirty-two patients with acquired hypogammaglobulinemia have had steatorrhea (16, 19, 22, 23, 28). Steatorrhea thus is a significant sign of the syndrome of acquired hypogammaglobulinemia. It has not always been possible to determine the time of onset of the sprue syndrome in relation to the development of hypogammaglobulinemia. In three of the reported cases (22, 23, 28) the steatorrhea developed after the manifestations of hypogammaglobulinemia. Steatorrhea has been exceeded in frequency only by splenomegaly with decreased blood cells (12 patients) (1, 10, 12, 13, 15, 19, 20, 28, 29) and by bronchiectasis (8 patients) (1, 12, 18, 20, 22, 27). Generally, patients with acquired hypogammaglobulinemia have not exhibited the cyclic leukopenia which has been described frequently in the congenital type.

Leukopenia was present in all of the twelve patients with splenomegaly. Two of them had their spleens removed (1, 15, 19) and in both the leukocyte counts became normal.

Acquired hypogammaglobulinemia probably is not the result of a defect of intestinal absorption. Such a defect leads to a decrease in all of the serum proteins. The slow rate of disappearance of I^{131}-labeled human gamma globulin from the blood of the patient in this report and others can be regarded as evidence that the low gamma globulin level is not a result of increased catabolism of this protein. Thus, the defect in acquired hypogammaglobulinemia, just as in the congenital form, seems to be an interference with the production of gamma globulin. The mechanism of the defect, however, may be different in these two forms of hypogammaglobulinemia.

Prophylactic replacement therapy by intramuscular administration of 0.1 gm. of pooled human gamma globulin per kg. of body weight at three-week intervals appears to have been successful in preventing bacterial infections in children with congenital hypogammaglobulinemia. Prophylactic therapy with antimicrobials may have a similar effect. In acquired hypogammaglobulinemia in adults, therapy with pooled human gamma globulin has often failed to protect the patient (1, 11–13, 21, 25, 27). Martin and his associates suggested a prophylactic regimen of higher doses of gamma globulin sufficient to keep the serum levels from 0.10 to 0.15 gm. per 100 ml. in excess of the levels in the untreated patient (13). They stated, however, that these doses provided a measure of protection for the patient only in combination with an antimicrobial drug. The experiences of the present writers with orally administered penicillin as the sole prophylactic

therapy have continued to be favorable. This probably is because pneumococci and streptococci are responsible for most infections in hypogammaglobulinemic patients. Such bacteria rarely become resistant to penicillin. Infections caused by staphylococci and enteric bacilli are surprisingly infrequent. At the present time the present writers are studying the oldest known surviving persons with congenital hypogammaglobulinemia, namely, two brothers, aged fifteen and twenty-one years. They have survived innumerable bouts of pneumonia, sinusitis, otitis media, and other infections largely because of prompt therapy of each new infection with antimicrobial drugs.

SUMMARY

Chronic steatorrhea preceded by four years the appearance of acquired hypogammaglobulinemia in a young man. The steatorrhea appeared to be the result of tuberculous mesenteric lymphadenitis. The almost complete absence of gamma globulin from this patient's serum proteins did not abolish delayed skin sensitivity to tuberculin. Recovery from the tuberculous infection was prompt in spite of the hypogammaglobulinemia.

A summary of the available literature on acquired hypogammaglobulinemia and the findings in this case suggest that acquired hypogammaglobulinemia often occurs as a sequel to a pathologic process involving the lymphatic system.

Steatorrhea is among the most frequent signs of the syndrome of acquired hypogammaglobulinemia.

SUMARIO

Esteatorrea y Probable Tuberculosis con Hipogammaglobulinemia Adquirida

Una esteatorrea crónica precedió de cuatro años la aparición de hipogammaglobulinemia en un joven. La esteatorrea parecía ser la secuela de una linfadenitis mesentérica tuberculosa. La falta casi absoluta de globulina gamma en las seroproteínas de este enfermo no abolió una cutisensibilidad retardada a la tuberculina. El restablecimiento de la infección tuberculosa fué rápido a pesar de la hipogammaglobulinemia.

Un sumario de la literatura accesible sobre la hipogammaglobulinemia adquirida y los hallazgos en este caso sugieren que dicha hipogammaglobulinemia se presenta a menudo como secuela de un proceso patológico que afecta el sistema linfático.

La esteatorrea figura entre los signos más frecuentes del síndrome de la hipogammaglobulinemia adquirida.

RESUME

Stéatorrhée et tuberculose probable avec hypogammaglobulinémie acquise

La stéatorrhée chronique a précédé de quatre ans une hypogammaglobulinémie chez un jeune homme. La stéatorrhée semblait être causée par une lymphadénite mésentérique. L'absence presque totale de gamma globulines dans les protéines sériques du malade n'abolissait pas le retard de l'allergie cutanée à la tuberculine. La guérison de l'infection tuberculeuse fut prompte en dépit de l'hypogammaglobulinémie.

Un résumé de la littérature médicale disponible relative à l'hypogammaglobulinémie et les résultats de l'étude de ce cas suggèrent que l'hypogammaglobulinémie acquise se manifeste souvent comme séquelle d'un processus pathologique intéressant le système lymphatique.

La stéatorrhée figure parmi les manifestations les plus fréquentes du syndrome de l'hypergammaglobulinémie acquise.

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ALLERGY TO ISONIAZID[1]

Successful Immunization in Two Cases

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INTRODUCTION

During the past few years isoniazid has been shown to be of value in the treatment of tuberculosis. Its efficacy is said to be one hundred times that of PAS (1), but it has the disadvantage that, when given alone, drug-resistant tubercle bacilli emerge and may present important therapeutic and epidemiologic problems.

Two types of untoward reaction to isoniazid are well known (2–5). The more common is a toxic one, examples of which are peripheral neuritis and convulsions. The less usual reaction is allergic (6, 7). Examples of this are drug fever, hepatitis, allergic pneumonia, and various types of dermatitis. It is generally assumed, because of positive skin tests and because of a history, signs, symptoms, and a time relationship compatible with allergic pathogenesis, that these reactions are caused by antibody reacting with the drug antigen. Such an antibody would be formed as a response to the drug (antigen) while the patient was taking it. Once such antibody levels were sufficiently high, the next dose of the drug (antigen) administered would cause clinical symptoms, presumably by combining with antibody with consequent cellular damage. The exact site of antibody formation in these cases is not known.

Under certain circumstances it may be particularly important for a patient to receive isoniazid although he is allergic to it. In these situations special effort should be made to enable the patient to tolerate the drug safely. This paper deals with two such cases, in which no other course seemed advisable, and immunization with isoniazid was attempted.

CASE REPORTS

Case 1. J. B. H., a 37-year-old white male, was admitted to the hospital on July 2, 1954, for treatment of pulmonary tuberculosis found on a routine chest film one month before. His only complaint was of a productive cough.

Physical examination showed a well-developed and -nourished man who did not appear ill. Examination of the eyes, ears, nose, and throat was negative. The lungs revealed no detectable abnormality. The heart size, sounds, and rhythm were normal. The blood pressure was 130 systolic, 80 diastolic in mm. of mercury. The remainder of the physical examination was unremarkable.

Laboratory findings were as follows: The hematocrit was 51, the leukocyte count was 8,000 with a normal differential pattern. The urine was negative. A roentgenogram showed exudative pulmonary tuberculosis of the left upper lobe with a cavity. The sputum was positive for acid-fast bacilli.

On July 8, 1954, a course of chemotherapy was started, 12 gm. of PAS and 300 mg. of isoniazid being given daily by mouth. On August 8, the patient complained of pleuritic pains, and a roentgenogram of the chest revealed a new area of infiltration in the left lower lung field. On August 12, the patient developed a fever of 102°F. The following day, coincident with a temperature of 103°F., a miliary pruritic eruption appeared on the chest,

[1] From the Department of Medicine, The George Washington University School of Medicine, Washington, D. C., and the Veterans Administration Center, Martinsburg, West Virginia.

then spread over the entire body. It became vesicular, then oozed and crusted. Large tender cervical lymph nodes became palpable, and the liver enlarged almost to the iliac crest. The patient became jaundiced. The spleen could not be outlined clearly although some observers believed that it was firm and enlarged. The leukocyte count rose to 15,000 with a shift to the left: 3 per cent myelocytes, 22 per cent bands and metamyelocytes, 19 per cent polymorphonuclear cells, 12 per cent eosinophils, 31 per cent lymphocytes, 7 per cent large lymphocytes, and 6 per cent monocytes. The urine remained normal. Blood urea nitrogen was 14 mg. per 100 ml. Heterophilic agglutinations were negative. Serum bilirubin was 3.2 mg. per 100 ml. Cephalin flocculation was 2-plus; thymol turbidity, 14.7 units; serum albumin, 2.9 gm. per 100 ml.; serum globulin, 4.2 gm. per 100 ml. The zinc sulfate turbidity test was 18.3 units, and the bromsulfonphthalein test showed 14 per cent retention. Six weeks later the alkaline phosphatase was 5.8 units.

All drugs were discontinued on August 17, 1954. For two weeks the patient ran a fever of 103°F. daily. After that the eruption gradually faded and the fever subsided. Liver function studies and the peripheral blood picture returned to normal by the end of September. One month later the pulmonary infiltration in the left lower lobe had disappeared. On one occasion a test dose of PAS, given through error, produced within five minutes a generalized itch, and approximately an hour later an erythematous eruption and a fever of 103°F. These signs and symptoms rapidly subsided.

About this time, the patient was referred to the allergy clinic for evaluation of his eruption, hepatitis, fever, and exacerbation of pulmonary infiltration. His family history was negative for allergy. His first and only injection of penicillin had caused transient urticaria in 1953. He had received no other antimicrobial drug before the present illness. Patch tests to PAS and to isoniazid, performed at different times, were strongly positive with induration and vesiculation. The patch test to streptomycin was negative. On October 12, 1954, since intradermal tests and trial doses of streptomycin were negative, the patient was started on the usual streptomycin schedule of 1 gm. intramuscularly twice weekly.

Since no complication had developed by November, and another patch test to isoniazid was strongly positive, it was decided to try to immunize the patient with isoniazid. Accordingly, injectable isoniazid[1] containing 100 mg. per ml. was diluted in sterile physiologic saline so that dilution *A* contained 0.1 mg. per ml.; dilution *B*, 1 mg. per ml.; and dilution *C*, 10 mg. per ml.

Immunization was started with a dose of 0.1 ml. of dilution *A* subcutaneously and increased gradually daily, as shown in table 1. On January 17, after a dose of 0.1 mg., a pruritic eruption appeared about the site of injection. The dose was decreased and gradually raised again, the sites of injection being alternated between the two arms. No further complication occurred until February 17 when, with a dose of 0.35 mg., the same type of eruption appeared about the injection site. Doses were increased cautiously with the hope that the eruption might subside, but it continued to spread about each site of injection in enlarging areas. Therefore, on February 24 the dose was reduced from 0.65 mg. to 0.3 mg., and again gradually increased without complication. On March 19, with a dose of 0.7 mg., an eruption again was noticed about the site of injection. This time it disappeared upon maintaining the dose at the same level for three days. Thereafter, no complications occurred.

On March 24, a left upper lobectomy was performed. Since then all examinations of the sputum have been negative for acid-fast bacilli.

By April 20, the patient was receiving 5 mg. of isoniazid subcutaneously without untoward reaction. From then on he was given the medication orally, increasing gradually from 25 mg. once a day to a full therapeutic dose of 100 mg. three times a day.

The patient has been maintained on full doses of streptomycin and of isoniazid for eleven months. On roentgenographic examination the area of infiltration adjoining the lobectomy scar has steadily decreased, and the patient was discharged in October, 1955.

[1] Used as Nydrazid, manufactured by E. R. Squibb & Sons.

Case 2. F. J. H., a 42-year-old white male, was admitted to the hospital on August 3, 1954, with the complaints of fatigue, weight loss, and productive cough of several months' duration.

Physical examination revealed an emaciated, acerbic man, complaining about everything. His height was 5 feet, 5 inches; his weight, 90 pounds, 30 pounds below his normal weight. His temperature was 100°F., his pulse, 132; his blood pressure 95 systolic, 60 diastolic in mm. of mercury. Examination of the eyes, ears, nose, throat, and neck showed no abnormality other than dental caries. Examination of the lungs revealed dullness at both apices with tubular breath sounds and a few fine rales. Pectoriloquy was present. Except for tachycardia, examination of the heart was negative, as was the remainder of the physical examination.

The erythrocyte count was 4,000,000; the leukocyte count, 13,000, with a normal differential. No eosinophils were seen. Corrected erythrocyte sedimentation rate was 32 mm. per hour. A roentgenogram of the chest showed pulmonary tuberculosis with bilateral cavitation. The sputum contained acid-fast bacilli.

On August 9, six days after admission, the patient was put on a regimen of streptomycin, 1 gm. intramuscularly twice weekly, and PAS, 12 gm. daily. After three weeks of this therapy he still remained febrile and had not improved clinically. On September 7, isoniazid was added to the chemotherapeutic regimen.

Six days later, on September 12, a morbilliform erythematous eruption appeared, first on the face and neck, then on the trunk and extremities. The patient's temperature, which had averaged less than 100°F., rose to 101–102°F by mouth. A leukocyte count showed a leukocytosis of 26,000, with 25 per cent eosinophils, the presence of abnormal lymphocytes and monocytes. Lupus erythematosus cells were not found on repeated search. Liver function studies showed an icteric index of 12 and an alkaline phosphatase of 15.2 which rose one week later to 20. Cephalin flocculation was 3-plus; serum bilirubin, 3 mg. per 100 ml.; and bromsulfonphthalein retention, 12 per cent. Serum proteins were within normal limits.

All drugs were discontinued. By early November the leukocyte count had decreased to 9,000 with 2 per cent eosinophils. No abnormal forms were noted. The eruption became exudative and progressed to a typical exfoliative dermatitis. Large cervical and axillary lymph nodes appeared, but the liver and spleen were at no time palpable.

In early December, three months after cessation of all three antimicrobial drugs, a roentgenogram of the chest showed evidence of progression of the disease on the left. On December 7, 1954, pneumoperitoneum was established, and fever finally subsided soon thereafter.

About this time the patient was seen for evaluation of drug allergy. His family and past histories were negative for allergy of any type. By March, 1955, the exfoliative dermatitis had healed sufficiently so that tests could be done. Patch tests performed at weekly intervals to full-strength streptomycin and to saturated solutions of isoniazid and PAS were all strongly positive with vesicle formation. Similar tests performed on tuberculous patients free of allergic drug symptoms all were negative.

Since the patient's tuberculosis had not improved sufficiently on rest alone, it was considered mandatory to give him chemotherapy. Since he appeared to be allergic to all three drugs, it was decided to attempt immunization with isoniazid. The same injectable preparation diluted in the same manner as in *Case 1* was used here. Immunization was started on April 2, 1955, with a dose of 0.01 mg. subcutaneously. The doses actually given are shown in table 2. They were well tolerated until April 26 when he received 1 mg. of isoniazid. About this time there appeared about the sites of injection red areas of pruritic dermatitis which were not infected. The dose was decreased to 0.2 mg. and gradually increased again without further complication until June 6 when the patient received 10 mg. subcutaneously.

On June 7 immunization was continued orally, starting with 12.5 mg. of isoniazid (one-fourth of a 50 mg. tablet) and increasing gradually as shown in table 2. No further setback occurred. However, on July 3 the patient complained of pains in his chest aggravated by eating and lying down, and relieved by sitting up. These were thought to be a complication

TABLE 1

SCHEDULE OF DOSAGE USED IN IMMUNIZATION OF CASE 1 WITH ISONIAZID

Date	Dilution	Route	Dose	Date	Dilution	Route	Dose	Date	Dilution	Route	Dose
1/ 8/55	A (0.1 mg. per ml.)	Subcutaneous	0.100	2/21/55	B	Subcutaneous	0.550	3/30/55	C	Subcutaneous	0.030
1/ 9/55			0.200	2/22/55			0.600	3/31/55			0.040
1/10/55			0.300	2/23/55			0.650	4/ 1/55			0.050
1/11/55			0.400	2/24/55			0.300	4/ 2/55			0.060
1/12/55			0.500	2/25/55			0.300	4/ 3/55			0.075
1/13/55			0.600	2/26/55			0.300	4/ 4/55			0.090
1/14/55			0.700	2/27/55			0.350	4/ 5/55			0.100
1/15/55			0.800	2/28/55			0.350	4/ 6/55			0.100
1/16/55			0.900	2/29/55			0.350	4/ 7/55			0.100
1/17/55*			1.000	3/ 1/55			0.400	4/ 8/55			0.120
1/18/55			—	3/ 2/55			0.400	4/ 9/55			0.125
1/19/55			0.500	3/ 3/55			0.450	4/10/55			0.125
1/20/55			0.550	3/ 4/55			0.450	4/11/55			0.150
1/21/55			0.600	3/ 5/55			0.500	4/12/55			0.150
1/22/55			0.650	3/ 6/55			0.500	4/13/55			0.200
1/23/55			0.700	3/ 7/55			0.500	4/14/55			0.200
1/24/55			0.750	3/ 8/55			0.550	4/15/55			0.250
1/25–1/31/55	Omitted through error			3/ 9/55			0.550	4/16/55			0.300
2/ 1/55			0.800	3/10/55			0.600	4/17/55			0.350
2/ 2/55			0.800	3/11/55			0.600	4/18/55			0.400
2/ 3/55			0.850	3/12/55			0.600	4/19/55			0.450
2/ 4/55			0.850	3/13/55			0.650	4/20/55			0.500
2/ 5/55			0.900	3/14/55			0.650	4/21/55		Oral	25 mg. once daily
2/ 6/55			0.900	3/15/55			0.650	4/22/55			25 mg. twice daily
2/ 7/55			0.950	3/16/55*			0.650	4/23/55			25 mg. twice daily

											A.M. mg.	Noon mg.	P.M. mg.
2/ 8/55			1.000	3/17/55			0.650	4/24/55			25	25	25
2/ 9/55			1.000	3/18/55			0.700	4/25/55			25	25	25
2/10/55	B (1 mg.	Subcuta-	—	3/19/55			0.700	4/26/55			25	25	25
2/11/55	per ml.)	neous	0.050	3/20/55			0.700	4/27/55			50	25	25
2/12/55			0.100	3/21/55			0.750	4/28/55			50	25	25
2/13/55			0.150	3/22/55			0.800	4/29/55			50	25	50
2/14/55			0.200	3/23/55			0.800	4/30/55			50	25	50
2/15/55			0.250	3/24/55			0.850	5/ 1/55			50	25	50
2/16/55			0.300	3/25/55			0.850	5/ 2-5/10/55			50	50	50
2/17/55*			0.350	3/26/55			0.900	5/10-5/17/55			75	75	75
2/18/55			0.400	3/27/55			0.900	5/17/55 to			100	100	100
2/19/55			0.450	3/28/55			0.950	present					
2/20/55			0.500	3/29/55	C (10 mg. per ml.)		0.025						

* Area of eruption at site of injection

TABLE 2

SCHEDULE OF DOSAGE USED IN IMMUNIZATION OF CASE 2 WITH ISONIAZID

Date	Route	Dilution	Dose	Date	Route	Dilution	Dose	Date	Route	Dose
4/12/55	Subcutaneous	A (0.1 mg. per ml.)	0.10 ml.	5/14/55	Subcutaneous	B (1 mg. per ml.)	0.85	6/15/55		25 mg. twice daily
4/13/55			0.20	5/15/55			0.90	6/16/55		25 mg. twice daily
4/14/55			0.30	5/16/55			0.95	6/17/55		25 mg. twice daily
4/15/55			0.40	5/17/55			1.00	6/18/55		25 mg. three times daily
4/16/55			0.50	5/18/55	Subcutaneous	C (10 mg. per ml.)	0.05 ml.	6/19/55		25 mg. three times daily
4/17/55			0.70	5/19/55			0.10	6/20/55		25 mg. three times daily
4/18/55			0.90	5/20/55			0.15		Oral	A.M. mg. / Noon mg. / P.M. mg.
4/19/55			1.00	5/21/55			0.20	6/21/55		50 25 25
4/20/55		B (1 mg. per ml.)	0.20 ml.	5/22/55			0.25	6/22/55		50 50 50
4/21/55			0.30	5/23/55			0.30	6/23/55		50 50 50
4/22/55			0.40	5/24/55			0.40	6/24/55		50 50 50
4/23/55			0.60	5/25/55			0.45	6/25/55		50 50 75
4/24/55			0.70	5/25/55			0.50	6/26/55		50 50 75
4/25/55			0.90	5/27/55			0.55	6/27/55		50 50 75
4/26/55*			1.00	5/28/55			0.60	6/28/55		50 50 75
4/27/55			0.05 ml.	5/29/55			0.65	6/29/55		75 75 75
4/28/55			0.10	5/30/55			0.70	6/30/55		75 75 75
4/29/55			0.15	5/31/55			0.75	7/ 1/55		100 75 75
4/30/55			0.20	6/ 1/55			0.80	7/ 2/55		100 75 75
5/ 1/55			0.25	6/ 2/55			0.85	7/ 3/55		100 75 75
5/ 2/55			0.30	6/ 3/55			0.85	7/ 4/55		Omitted through error

5/ 3/55			0.35	6/ 4/55			0.90	7/ 5/55	Omitted through error			
5/ 4/55			0.40 ml.	6/ 5/55			0.95	7/ 6/55		50	50	50
5/ 5/55			0.45	6/ 6/55			0.95	7/ 7/55		50	50	50
5/ 6/55			0.50	6/ 7/55			1.00 ml.	7/ 8/55		50	50	50
5/ 7/55			0.55	6/ 8/55	Oral		12.5 mg.	7/ 9/55		50	50	50
5/ 8/55			0.60	6/ 9/55			12.5 mg.	7/10/55		50	50	50
5/ 9/55			0.65	6/10/55			12.5 mg. twice daily	7/11/55		75	75	75
5/10/55			0.70 ml.	6/11/55			12.5 mg. twice daily	7/12/55		75	75	75
5/11/55			0.70	6/12/55			12.5 mg. three times daily	7/13/55		75	75	75
5/12/55			0.75	6/13/55			12.5 mg. three times daily	7/14/55 to present		100 (full dosage)	100	100
5/13/55			0.80	6/14/55			25 mg. twice daily					

* Allergic dermatitis

of his pneumoperitoneum. After a lapse of two days the immunizing doses of isoniazid were continued until July 15 when he was taking a full therapeutic dose of 100 mg. three times a day without untoward reaction.

The patient has been maintained on this dose for ten months at the time of writing. A roentgenogram of the chest in July, 1955, showed decrease in the size of both cavities, although his sputum was still positive for acid-fast bacilli in June. The patient has gained 12 pounds.

Since isoniazid-resistant tubercle bacilli may present problems when isoniazid is given alone, immunization with streptomycin is now planned. A recent patch test showed exquisite sensitivity to this drug.

COMMENT

Immunization when the antibody is not demonstrated by *in vitro* techniques is always difficult to prove. This is particularly so when the hypersensitivity has to be judged by patch tests which cannot be calibrated as accurately as intradermal or scratch tests. In both the reported cases the patients were allergic to more than one drug. It would have been advisable in both cases to give test doses of each drug separately to see what features of the generalized reaction were caused by each drug. However, the administration of test doses was considered too hazardous in these patients who had almost died from their hypersensitivity.

The first patient gave a positive patch test to PAS, and an inadvertent dose of PAS produced fever and a transient eruption. Hence, there can be little doubt about his hypersensitivity to PAS. A patch test to streptomycin was negative and he tolerated immediately the usual dose of streptomycin (1 gm. twice a week); he was not considered to be allergic to this drug. To isoniazid, however, he gave a positive patch test. The present writers have tested routinely many patients receiving isoniazid without ever encountering a positive reaction except in patients frankly allergic to it. The likelihood that this patient was allergic to isoniazid is borne out by the fact that on three separate occasions he had large local reactions about the site of injection. These local reactions were quite characteristic in that they were red, swollen, lasted forty-eight hours or more, and were followed by a fine desquamation of the skin every time that they occurred. The solution used for immunization was not contaminated, as shown by sterile culture and the fact that the same solution was tolerated later by the same patient after repeating the dose several times. This series of events is seen commonly in immunizing patients against pollen and other antigens.

It is unfortunate that the patient was not seen in the allergy department at the onset of the sudden spread of his pulmonary infiltration. It was the impression of the radiologists that the infiltration resembled the pattern seen in Löffler's syndrome rather than a spread of the tuberculosis. The eosinophilic count rose from 1 to 12 per cent at this time, but it was not repeated frequently nor was the sputum examined for the presence of eosinophils. Therefore, the point cannot be proved.

In the second case the patient had received usual doses of PAS and streptomycin for four weeks before isoniazid was added. Six days later an eruption appeared, his fever rose, he looked much sicker than before, and his leukocyte count rose to 26,000 with abnormal lymphocytes and 25 per cent eosinophils. Patch tests to all three drugs were strongly positive over several weeks. During attempted immunization this patient developed the same type of local reaction as did the patient in *Case 1*. The same and higher doses of the solution used for immunization were tolerated without local or generalized reaction upon repetition of dosage.

The great variability of drug reactions is well known. A drug not tolerated at one time may be tolerated some weeks, months, or years later. Conversely, some drugs, having

once caused an allergic reaction, may not be tolerated again for long periods, perhaps for a lifetime. Well-documented follow-up periods have lasted more than twenty years.

When drug reactions do occur, it frequently is not possible to prove whether the reaction is toxic or allergic. Fortunately, in the present cases it was possible to get typically positive patch tests, indicating that an antibody was almost surely involved. Obviously, it would have been desirable to find a patient allergic to isoniazid alone. However, no such patient was available.

There is an impression at the clinical level that PAS actually enhances the possibility of a hypersensitivity reaction to any other drug administered at the same time. Instances of patients allergic to PAS who also become allergic to streptomycin are not rare, and in *Case 2* the patient became hypersensitive to isoniazid as well. However, the reaction is probably, at least in part, specific, for cases are found, as in *Case 1*, in which the patient is frankly allergic to PAS and isoniazid, yet not to streptomycin.

The 2 patients of the present study are thought to be allergic to isoniazid (even though they are allergic to another drug as well) because of the following facts:

Each had symptoms that have been shown to be caused by antigen-antibody reactions in other cases.

The symptoms occurred after an interval suitable for the formation of antibodies.

Patch tests to isoniazid were markedly positive. They are negative in most patients who receive this drug.

During "immunization" the patients developed on several occasions marked local or generalized reactions of the type seen in allergic patients when an overdose of antigen has been given.

It is impossible to state at the present time whether these patients developed true immunity to isoniazid or whether they merely developed a tolerance. *In vitro* studies were not done, and generally they are unrewarding when drug antigens of this type are used. Further studies will be attempted to elucidate this point.

The schedules used in immunization are not to be taken necessarily as models. They are merely a first attempt in dealing with a new drug. With more experience using isoniazid a shorter and more practical schedule may be worked out. Both of these patients were overwhelmingly allergic, and every precaution had to be taken.

It is believed that the benefit of isoniazid should not be denied any hospitalized tuberculous patient allergic to the drug until immunization has at least been attempted. Special training in allergy probably is not necessary for this type of immunization. However, the ability to differentiate allergic from other complications makes the task easier for the immunizing physician. All the doses given are set down in the tables so that an extremely graduated approach may be seen.

The present writers were unable to find in the literature any previous case of attempted immunization with isoniazid.

SUMMARY

Two cases of serious allergy to isoniazid are reported. Both patients were immunized successfully. The details of immunization are given and discussed. It is believed that allergy to isoniazid in hospitalized tuberculous patients does not necessarily preclude use of the drug. In these cases immunization should at least be attempted. No previous report of immunization to isoniazid was found in the literature.

SUMARIO

Alergia a la Isoniacida: Exito de la Inmunización en Dos Casos

Preséntanse 2 casos de alergia grave a la isoniacida. Ambos enfermos fueron inmunizados con éxito. Se exponen y discuten los pormenores de la inmunización. Parece que la alergia a

la isoniacida en los tuberculosos hospitalizados no veda forzosamente el empleo de la droga. En estos casos, debe a lo menos probarse la inmunización. En la literatura no se encontró ninguna comunicación anterior sobre la inmunización.

RESUME

Allergie à l'isoniazide: Succès de l'immunisation dans deux cas

Deux cas d'allergie grave à l'isoniazide sont rapportés. Ces deux cas furent immunisés de façon satisfaisante. Les détails de l'immunisation sont exposés et commentés. Les auteurs estiment que l'allergie à l'isoniazide chez le tuberculeux hospitalisé ne prévient pas nécessairement l'usage du médicament. Dans ces cas, l'immunisation devrait au moins être tentée. Aucun antérieur rapport sur l'immunisation à l'égard de l'isoniazide n'a été trouvé dans la littérature médicale.

Addendum

During the writing of this paper, a third patient has been "immunized" against isoniazid. Patch tests were strongly positive to streptomycin and to isoniazid, negative to PAS. The course of immunization was stormy. The patient developed three large local reactions and three generalized reactions. He has been maintained on full doses of isoniazid for six months and is now on leave for a month from the hospital.

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(6) Mellette, S. J., and Agress, H.: Toxic reaction to PAS and isoniazid accompanied by leukopenia and atypical lymphocytosis, Am. Rev. Tuberc., 1954, *69*, 824.

(7) Kalbian, V. V.: Isonicotinic acid hydrazide hypersensitivity with pyrexial reaction: Report of a case, Tubercle, 1954, *35*, 221.

Editorial

WILL HISTORY REPEAT?[1]

Some of the issues of today in our field of endeavor and some of the problems link up closely with some of the issues and problems of the past, with which comparisons may be made. One of these refers to infectiousness or contagion of consumption. This has struck fear over the ages. To Aristotle, those who cared for consumptives not infrequently developed consumption. Galen thought it unsafe to spend an entire day in the presence of the disease. Van Swieten in the Seventeenth Century wrote of a case in which the "final kiss of a dying wife left a permanent bald spot in her husband's beard." And, later, G. Sand, paramour of Chopin, recounted the latter's problems when his hemorrhages revealed his diagnosis and when that great man of music was trundled to the docks in a wheelbarrow—a victim of public fear.

As a far-away echo of this fear, medical journals nowadays frequently recount in case history fashion the story of the development of tuberculosis among hospital personnel—physicians, nurses, technicians, and others. In this situation, sanatorium administrators find a marked deterrent to recruitment of staff, and nursing departments regularly carry unfilled positions because trainees have heard this distressing news. Anesthetists shy away from tuberculous patients at operation, and laboratory directors tear their hair while putting forth almost frantic efforts at times to obtain or replace personnel. Extensive efforts to ensure safety become the trademark of the day.

This phenomenon has appeared only in fairly recent years and probably represents a product of the shift from employment, predominantly, of former tuberculous patients. In earlier times the "fall-out" from tuberculosis among employees was deemed to represent exacerbation of pre-existing disease; now it stands as newly acquired disease.

Until late in the 1700's this attitude of fear and respect characterized the thoughts and words of both layman and physician on the subject. About this time something happened to medical thinking, and physicians began to talk of the "foolish idea of contagion" (Bichat). Several experimenters (Kortum, 1789; Hébréard, 1802; Samade, 1805) inoculated material, especially from tuberculous cervical adenitis, into animals and in one case into man (Lepelletier, 1830), with no ill result, and thus they confirmed the physician in his contention of noninfectiousness—a position he held for more than seventy-five years and stoutly defended even against the convincing experiments of Villemin. Pidoux, in the French Academy as late as 1867, expressed the thought of that day (published in 1873):

"What a misfortune," he exclaimed, "should things result thus! Social economy, public and private hygiene, prophylaxis, medicine—all their aspirations, all their efforts con-

[1] Presented as the address of the President of the American Trudeau Society at the annual meeting of the Society, held jointly with the National Tuberculosis Association in New York, May 21–24, 1956.

demned in advance! The poor consumptives sequestered like the bearers of plague! Family tenderness at war with fear, and self preservation face to face with a malady, which, because of its prolonged course and homicidal atmosphere, would wear out devotion! If consumption is contagious, we dare not say it aloud."

Fortunately, Koch's masterly demonstration laid the dust on this question, apparently forever.

Current developments in the treatment of tuberculosis likewise bear on this issue. Better treatment yields earlier discharge from the sanatorium and, with it, a tendency to treat patients at home. This becomes a real issue. Although the effectiveness of drug treatment increases steadily as we move along in time, prolonged and uninterrupted application of drugs produces a disappearance of tubercle bacilli from the sputum in four to six months in only three-fourths of patients. Physicians are increasingly convinced that drug therapy allows considerable relaxation in the scheme of bed rest so essential to recovery in the pre-drug era. Earlier discharge of patients who do well represents a safe scheme. In North Carolina until about two years ago the average stay of the patient in the hospital was something more than twelve months—now it approximates eight months.

However, home care for patients with tubercle bacilli in the sputum poses problems fraught with danger. Drug treatment under optimal conditions *will* usually render the patient's sputum negative for tubercle bacilli, but, in the most favorable circumstances, does so only within a span of several months. During this period infection of others is an easy possibility provided contact continues, as witnessed a while ago when a patient with "positive" sputum left the institution. Twelve weeks later the infant son in the family entered with tuberculous meningitis. Added irony lies in the demonstration of drug resistance of the bacilli in both instances.

In some homes, conditions do exist that allow proper isolation and care for patients, but, categorically it seems to me that patients with tubercle bacilli in the sputum always belong in the hospital unless special indices point to the contrary. After all, tuberculosis stands in the forefront of the list of diseases with public health implication. It spreads from those who have it to at least some who do not. As long as the patient with active tuberculosis remains in his home, waiting out the course of chemotherapy—waiting with his germs and his children—he remains a breeder of new cases and a continuing threat to his family and associates. From him new cases may develop—with all the grief, all the personal and family tragedy, and all the expense which follow in the wake of this disease. How important, therefore, to protect the susceptibles from the carriers! The consumptive on the loose becomes everybody's business, for he continues to distribute his bacilli.

All understand that gradations of exposure present themselves and depend upon the density of population of bacilli in the milieu. This can be modified by the numbers of bacilli produced, the intimacy of the exposure and the frequency of its repetition, the duration of the contact, and the sanitary arrangements that prevail. The "positive" patient properly taking his prescribed drugs must, on

the average, present fewer bacilli to his surroundings and surely does so over a shorter period than the patient with similar disease not so treated. *But one may well fear possible infection as long as situations exist which support exposure.* One should not be satisfied with a "half-safe" state if contacts can be broken.

You may think that, in the vernacular, I "accentuate the positive." This is done because we do face this dilemma. On the one hand we champion efforts in the institutions to protect employees from developing tuberculosis and do so in the certain knowledge that we deal with an infectious disease. At the same time, we condone or encourage housing of the positive patient at home—positive in diminishing ratio it may be, at least for the first several months of his period of treatment.

What position should we take? How can we support the one position and at the same time cling to the other? Do we think in all honesty that protective measures should be vigorously extended to all contacts? No man can serve two masters. Was the wry old orderly correct when he said: "It looks like tuberculosis ain't as ketchin' as it used to be."? A verse of scripture seems to apply at the moment which says "Whatsoever things are true, whatsoever things are honorable, . . . whatsoever things are of good report; if there be any virtue, if there be any praise, think on these things."

These questions constitute background material—material for work now and for work in the years ahead. We shall need to think on them lest we lapse into a state which fails to reckon fatefully and well upon the infectiousness of this disease.

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Notes

ANTIMICROBIALLY ACTIVE CONCENTRATIONS OF SALIZID® AND ISONIAZID IN THE BLOOD

Steenken and associates[1] reported that Salizid,® a hydrazone of isonicotinic acid hydrazide (isoniazid) with salicylaldehyde, is similar to isoniazid in its *in vitro* tuberculostatic effect and in its action on experimental tuberculosis in the guinea pig, mouse, and rabbit. Katz and associates[2] reported clinical results with Salizid and compared them with isoniazid. At the same dosage, 200 to 400 mg. per day, they found it to be as effective as isoniazid in thirteen original treatment and twenty-four re-treatment patients. When

TABLE 1

MICROBIOLOGIC SERUM ASSAY FOR ISONIAZID ACTIVITY SIX HOURS AFTER TEST DOSE

Patient	Drug	Dosage	Concentration Achieved	Salizid Concentration / Isoniazid Concentration × 100
			γ per ml.	
1	Salizid	300 mg. (5 mg. per kg.)	0.80	
	Isoniazid		2.40	33
2	Salizid	300 mg. (5 mg. per kg.)	1.20	
	Isoniazid		1.60	75
3	Salizid	500 mg. (7 mg. per kg.)	<0.40	
	Isoniazid		1.20	<33
4	Salizid	600 mg. (8 mg. per kg.)	1.20	
	Isoniazid		3.20	38
5	Salizid	400 mg. (7 mg. per kg.)	1.20	
	Isoniazid		2.40	50

Average < 46 per cent

peripheral neuritis because of isoniazid appeared, Salizid could be substituted in the same dose without further ill effects and, indeed, the neuritis subsided in most instances. Salizid was tolerated in several epileptic patients who experienced increase in frequency of seizures when they received isoniazid. In a further paper,[3] these workers stated that Salizid in a dosage of 1,000 mg. per day produced no peripheral neuritis in fifty original treatment patients. In the majority of patients whose peripheral neuritis was caused by isoniazid, the substitution of Salizid for isoniazid at the same dosage or at 1,000 mg. per day was associated with remission of the neuritis in twelve of fifteen patients.

The clinical and experimental work on Salizid[1-3] and the isoniazid content of Salizid suggests that on a weight basis Salizid should be approximately one-half as effective as isoniazid by itself.[5] This assumption was tested by comparing the values for isoniazid

[1] STEENKEN, W., JR., *et al.*: Proc. Soc. Exper. Biol. & Med., 1954, *37*, 194.

[2] KATZ, S., *et al.*: Tr. Thirteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1954, p. 374.

[3] MCCORMICK, G., *et al.*: Tr. Fourteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 332.

[4] HART, J. J. D., *et al.*: Antibiot. & Chemother., 1954, *4*, 803.

[5] There is no evidence that the salicylaldehyde moiety has any antimicrobial activity.

activity, in the blood, determined microbiologically, following identical doses of Salizid[6] and isoniazid.

Serum microbiologic assays for "isoniazid" activity[7] were performed in 5 tuberculous patients. The determinations were done under identical conditions utilizing the same dose of Salizid and isoniazid. The results are presented in table 1. The patients receiving Salizid achieved serum "isoniazid" concentrations from below 33 to 75 per cent of those attained when they received isoniazid. The average for the 5 patients was below 46 per cent.

The antimicrobial action of isoniazid resides mainly or entirely in the free form of the compound[8] which is the fraction measured by microbiologic assay. Although Salizid may be substituted for isoniazid, it must be remembered that it is approximately one-half as effective on a weight for weight basis. Therefore, indiscriminate substitution of Salizid for isoniazid, employing similar dosages, may not lead to comparable therapeutic results. The lower incidence of neurotoxicity following Salizid may be due in part to the lower concentration of antimicrobially active isoniazid achieved on administration of this drug.

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I. Kass
W. F. Russell, Jr.

May 23, 1956

[6] Supplied by Dr. George Mast, Nepera Chemical Company, Yonkers, New York.

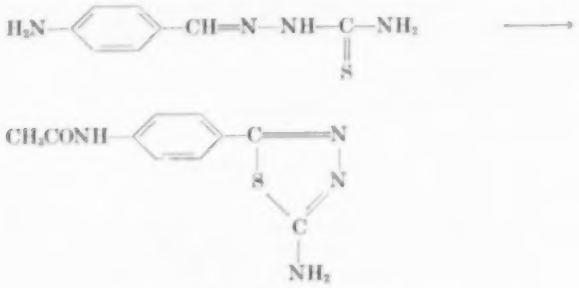
[7] Mandel, W., *et al.*: Proc. Soc. Exper. Biol. & Med., 1956, *91*, 409.

[8] Hughes, H. B., *et al.*: Tr. Fourteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 217.

THE METABOLIC PRODUCTS OF HINCONSTARCH

The new antituberculous drug, hinconstarch, is made by condensing periodate-oxidized starch through its potential aldehyde groups with an equimolar mixture of isoniazid and *p*-aminobenzalthiosemicarbazone (*p*ABT).[1, 2] It is an effective agent in experimental tuberculosis of mice and guinea pigs whether the infecting bacilli are susceptible or resistant to isoniazid,[2, 3] and it appears, therefore, that both active principles of the molecule are absorbed. It has also been shown to be active in human pulmonary tuberculosis.[4]

The present writers have now found in the urine of patients undergoing hinconstarch therapy, isonicotinic acid, isonicotinyl glycine, and 5-*p*-acetamidophenyl-2-amino-1:3:4-thiadiazole (*B*), the last being derived from the thiosemicarbazone (*A*) by oxidative cyclization and acetylation.



These findings provide direct chemical confirmation that both active principles of the drug are absorbed.

Several considerations suggest however that the processes of digestion do not simply liberate isoniazid and *p*ABT from the hinconstarch. First, the amount of the thiosemicarbazone combined in hinconstarch which can be administered to patients without producing toxic symptoms is very much greater than the amount of parent thiosemicarbazone or its acetyl derivative, i.e., amithiozone,[7] which can be given. Second, thiostarch, the corresponding polymer obtained when oxystarch is condensed with thiosemicarbazide, exerts a suppressive effect on experimental tuberculosis, whereas thiosemicarbazide itself has no such effect.[3] Furthermore, it has been shown that similar polymers derived from oxystarch break down under mild conditions in the test tube to yield derivatives of glyoxal and erythrose.[5] This suggests that the linkages formed during the condensation are not the most readily cleaved.

The blood of patients receiving the drug has been shown to contain 2–3 γ per ml. (estimated as isoniazid) of "hydrolyzable hydrazine," two and one-half hours after the

[1] Barry, V. C., *et al.*: Proc. Roy. Irish Acad., 1954, *57B*, 47.
[2] Barry, V. C.: J. Irish M. A., 1956, *38*, 68.
[3] Barry, V. C., *et al.*: Brit. J. Urol., 1955, *27*, 35.
[4] Wolinsky, E., and Steenken, W., Jr.: Am. Rev. Tubere., 1956, *73*, 72.
[5] Wolinsky, E., and Steenken, W., Jr.: Tr. Fifteenth Conference on Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, February, 1956 (in press).
[6] Barry, V. C., *et al.*: Am. Rev. Tubere., 1956, *73*, 219.
[7] Hinshaw, H. C., and McDermott, W.: Am. Rev. Tubere., 1950, *61*, 145.
[8] Barry, V. C., and Mitchell, P. W. D.: J. Chem. Soc. London, 1953, 3610.

patients had received an oral dose of 1–1.2 gm.[9] Unlike isoniazid, which is rapidly absorbed and excreted, the blood concentrations of "hydrolyzable hydrazine" from hinconstarch remain steady for long periods; and early morning values, sixteen hours after the last dose in patients receiving 3.0 gm. per day, are usually not below 1 γ per ml. and are frequently much higher. It should be emphasized, however, that the *p*-dimethylaminobenzaldehyde reagent used in this method[10] estimates both isoniazid and thiosemicarbazone.

Identification of metabolites: Urine was collected from patients over 24-hour periods, preserved by addition of thymol or chloroform, and stored at 0°C. Aliquots were reduced to one-tenth volume by evaporation under reduced pressure and centrifuged. Portions of the supernatants were applied to paper chromatograms and developed in isopropanol-water (80:20). Isonicotinic acid and isonicotinyl glycine were identified by means of the spray reagents of Cuthbertson and associates.[11] The presence of isonicotinyl glycine was further confirmed by acid hydrolysis of the eluate of the appropriate area of the chromatogram. Chromatography of the neutralized hydrolysate showed the presence of isonicotinic acid and glycine (ninhydrin spray). In contrast to the experience of Hughes[12] with urine of patients receiving isoniazid, no acetylisoniazid was detected in the urine of the patients. At least one-fifth of the administered dose of hinconstarch in terms of its isoniazid content was present in the urine as isonicotinic acid and isonicotinyl glycine. This is approximately the same proportional recovery of these substances as has been found in patients receiving orally 200 mg. of isoniazid alone per day.

When chromatograms of the urine concentrates were viewed under a short-wave U.V. lamp (Filter OX 7, 300 mμ), a dark blue fluorescent spot was observed (Rf. in isopropanol-water, 0.55). No such spot occurred in normal urine or in the urine of patients who received isoniazid alone. The fluorescent material did not migrate on paper electrophoresis at neutral or alkaline pH but became positively charged and migrated at pH 1. It was extracted by ether from the basified urine and the extract was dried over anhydrous sodium sulfate. The extract showed a dark blue fluorescence in ultraviolet light; on concentration the solution became yellow and exhibited a yellow fluorescence. It was adsorbed on an alumina column forming a dark blue fluorescent band (observed in the ultraviolet) and was eluted with ethanol-ether (50:50).

The fluorescent material in this solution was shown to be 5-*p*-acetamidophenyl-2-amino-1:3:4-thiadiazole, an authentic specimen (m.p. 237–239°) of which was synthesized by the method of Bernstein and co-workers.[13] The authentic thiadiazole hydrochloride solution in ethanol showed the same fluorescent properties as the metabolite in ultraviolet light. On chromatography in acidic, basic, and neutral solvents, both the thiadiazole and the unknown gave identical dark blue fluorescent spots with the following Rf. values: isopropanol:water (80:20), 0.55; butanol:acetic acid:water (50:10:40) (butanolic phase), 0.60; ethanol:concentrated ammonia (100:1), 0.57. They were readily differentiated from *p*-acetamidobenzalthiosemicarbazone, which gave a dark absorbing area in ultraviolet light, and had the Rf. values of 0.50, 0.57, and 0.53, respectively, with the above solvents. Unlike thiosemicarbazones, both the synthetic material and the metabolite gave negative reactions for —C=S or —C—SH when chromatograms were sprayed with sodium azide and iodine.[14] They both migrated in the presence of copper

[9] Waldron Edward, D.: Unpublished results.
[10] Waldron Edward, D.: Irish J. M. Sc., 1956, *363*, 130.
[11] Cuthbertson, W. F. J., *et al.*: Biochem. J., 1953, *55*, 669.
[12] Hughes, H. B.: J. Pharmacol. & Exper. Therap., 1953, *109*, 444.
[13] Bernstein, J., *et al.*: J. Am. Chem. Soc., 1951, *73*, 906.
[14] Feigl, F.: Manual of Spot Tests, Academic Press, New York, 1943, p. 180.

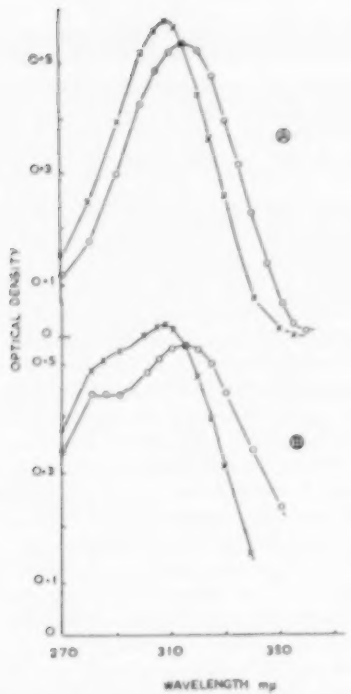


FIG. 1. Ultraviolet absorption curves *A:* Authentic 5-*p*-acetamidophenyl-2-amino-1:3:4-thiadiazole, 4.6×10^{-5} M. *B:* Purified urinary extract from hinconstarch patient. Solvents: ×——× Ethanol: 2N HCl (50:50), ○——○ Ethanol: water (50:50).

sulfate when chromatograms were developed with isopropanol:water, whereas the thiosemicarbazone, which forms a copper complex, remained at the origin.

In addition to the thiadiazole, the eluate from the alumina column was shown, by its ultraviolet absorption in neutral and acid solution, to contain a material with maximal absorption at 280 mμ (figure 1). The nature of the material has not been established.

The concentration of the thiadiazole in the urine was estimated by spot dilutions on chromatograms, comparing urine concentrates with standard solutions of synthetic material. Patients receiving 1.2 gm. of hinconstarch (approximately equivalent to 300 mg. of *p*ABT) daily excreted 10 to 20 mg. of thiadiazole. No thiadiazole could be detected in normal urine to which the thiosemicarbazone (or its acetyl derivative, amithiozone) had been added, and which had been treated as above, indicating that it is unlikely that the oxidative ring closure took place in the voided urine.

Spinks[15] found no evidence of thiadiazole in the urine of rabbits fed with various thiosemicarbazone derivatives. The low yield of thiadiazole obtained from the urine of patients receiving doses of hinconstarch possibly indicates that, as in the case of 4-ethylsulfonyl-benzalthiosemicarbazone,[16, 17] most of the thiosemicarbazone undergoes hydrolysis *in vivo* to thiosemicarbazide which is then metabolized further.

[15] SPINKS, A.: Brit. J. Pharmacol., 1951, *6*, 35.
[16] POPPE, H., *et al.*: Arch. f. exper. Path. u. Pharmakol., 1954, *224*, 145.
[17] HINRICHS, K., *et al.*: Arch. f. exper. Path. u. Pharmakol., 1954, *222*, 513.

Effect of acid or gastric juice digestion on hinconstarch: On treating hinconstarch with 0.05N HCl or normal gastric juice at 37°C. for several hours, a considerable part of the material dissolved. A little free *p*ABT was extractable from this solution with butanol. The major part of the dissolved substance was obtained as an amorphous precipitate on neutralizing with sodium bicarbonate, leaving a clear yellow filtrate. The colored material in this filtrate diffused completely through a viscose casing, indicating that it had a relatively low molecular weight. Both the precipitate and the filtrate gave rise to a similar series of fluorescent spots when chromatograms were viewed under ultraviolet light. The predominating spot was orange with approximately the same Rf. (0.50 in isopropanol: water) as *p*ABT, which has, on the contrary, however, a light blue fluorescence. The orange fluorescent material gave a positive reaction in the sodium azide-iodine test, and was retained at the origin when chromatograms were developed in the presence of copper sulphate, indicating that it contained the unchanged $=N \cdot NH \cdot CS \cdot NH_2$ group.

These digestive treatments failed to yield a material which behaved chromatographically like 5-*p*-acetamidophenyl-2-amino-1:3:4-thiadiazole or its deacetylated analogue obtained by hydrolysis. The results indicate that dilute acid causes depolymerization of the hinconstarch, but oxidative ring closure of thiosemicarbazone probably does not take place in the stomach.

In summary: The antitubercuious activity of hinconstarch is discussed and the identification, in the urine of patients undergoing treatment with this drug, of isonicotinic acid, isonicotinyl glycine, and 5-*p*-acetamidophenyl-2-amino-1:3:4-thiadiazole is described.[18]

LABORATORIES OF THE MEDICAL RESEARCH COUNCIL OF IRELAND
TRINITY COLLEGE
DUBLIN, IRELAND

VINCENT C. BARRY
DEIRDRE WALDRON EDWARD[19]

June 20, 1956

[18] Recently we have found, in addition, free isoniazid in one urine specimen, and diisonicatinylhydrazine in another. Acetylisoniazid has not been found up to the present time.

[19] Lasdon Foundation Research Fellow of University College, Dublin.

ON THE PHYSIOLOGIC DISPOSITION OF CYCLOSERINE IN EXPERIMENTAL ANIMALS[1]

In certain respects cycloserine (D-4-amino-3-isoxazolidinone) occupies an anomalous position in the chemotherapy of tuberculosis. Several investigators[2–5] have found that the compound has a reasonably high order of tuberculostatic activity *in vitro*. In keeping with this, the drug has exhibited significant beneficial effects in the treatment of naturally acquired pulmonary tuberculosis in man[6–9] and the induced disease in the rhesus monkey.[10] In contrast, essentially no activity or very limited activity has been demonstrated i exnperimental infections of mice,[3, 5, 11] guinea pigs,[5, 12, 13] and rabbits.[14]

In search for an explanation for the diverse findings referred to above, it was thought that they might rest on differences in the manner in which various animals metabolize or excrete cycloserine. This suggestion led to systematic studies of the clearance of this drug from the circulating blood of the mouse, guinea pig, rabbit, and monkey, following administration of doses which have been used in the successful treatment of simian infections and relatively unsuccessful treatment of rodent infections. The results of these studies are summarized here.

Clearance of cycloserine from blood: This part of the investigation involved experiments with 40 white mice, 12 guinea pigs, 3 rabbits, and 6 rhesus monkeys. The mice were of the Schwing strain, males, weighing from 20 to 34 gm.; the guinea pigs were young albino females, weighing 350 to 430 gm.; the rabbits were New Zealand White females, weighing 2.0 to 2.1 kg.; the monkeys were young animals weighing 4.3 to 4.7 kg. Except for the monkeys, which were fed their regular ration, food was withheld from all animals during the

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[1] This investigation was supported in part by a research grant (E 509) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service.

[2] Cummings, M. M., *et al.*: Tr. Fourteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 321.

[3] Cuckler, A. C., *et al.*: Antibiot. & Chemother., 1955, *5*, 191.

[4] Cummings, M. M., *et al.*: Antibiot. & Chemother., 1955, *5*, 198.

[5] Steenken, W., Jr., and Wolinsky, E.: Am. Rev. Tubere., 1956, *73*, 539.

[6] Epstein, I. G., *et al.*: Tr. Fourteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 326.

[7] Epstein, I. G., *et al.*: Antibiot. Med., 1955, *1*, 80.

[8] McLean, R.: Tr. Fifteenth Conference on the Chemotherapy of Tuberculosis, 1956 (in press).

[9] Lester, W., Jr., *et al.*: Am. Rev. Tubere., 1956, *74*, 121.

[10] Schmidt, L. H.: Unpublished data, report to Veterans Administration Central Committee, Conference on Cycloserine, October 4, 1955, Washington, D. C., p. 2.

[11] Welch, H., *et al.*: Tr. Fourteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 314.

[12] Patnode, R. A., *et al.*: Am. Rev. Tubere., 1955, *72*, 117.

[13] Patnode, R. A., *et al.*: Am. Rev. Tubere., 1955, *72*, 856.

[14] Raleigh, J. W., *et al.*: Quarterly Progress Report, Veterans Administration, Army, and Navy Study on Chemotherapy of Tuberculosis, July, 1955, p. 25.

experimental periods. Water was offered ad libitum. Cycloserine,[15] as a freshly prepared solution in physiologic saline, was administered to all animals as a single intramuscular injection. The doses employed in these experiments ranged from a low of 50 mg. per kg. of body weight for the monkeys to a high of 400 mg. per kg. of body weight for the mice.

At various intervals after administration of the drug, blood specimens were obtained and analyzed for cycloserine according to a method described by Jones.[16] These blood samples, usually 1.5 ml. or less, were obtained from monkeys by venipuncture and from rabbits by successive cardiac puncture; blood from mice and guinea pigs was withdrawn directly from the exposed heart of the anesthetized animal. In order to obtain sufficient blood from mice for cycloserine assays, the blood from 4 animals was pooled for each analysis.

Recovery of drug in urine: Urine specimens from 12 mice, 4 guinea pigs, 3 rabbits, and 2 monkeys were collected into iced vessels over sodium carbonate for a 24-hour period prior to drug administration and for various intervals thereafter up to the twenty-fourth hour for the small animals and up to 48 hours for the monkeys. Aliquots of each urine specimen, diluted with at least ten parts of one per cent sodium carbonate, were assayed for cycloserine according to the method of Jones.[17]

Paper chromatographic methods[18] were employed in an attempt to detect urinary metabolites, other than unchanged cycloserine, which might react with the color reagent used for assay.

In figure 1 are shown the concentrations of cycloserine in the blood of different laboratory animals at various times after intramuscular administration of the drug. The marked species variations in the physiologic disposition of cycloserine are reflected in clearance values calculated from data shown in this chart. It will be noted that the peak concentration of cycloserine in the blood of all species, except the monkey, was reached within 30 minutes after injection and that the concentration declined nearly logarithmically from this value. Differences in clearance rates among the laboratory animals can be compared conveniently if the drug half-life values, i.e., the time required for the drug concentration in the blood to reach a value one-half that determined previously, are considered. The mean values are as follows: mouse, 23 minutes; guinea pig, one hour; rabbit, 2½ hours; monkey, 7¾ hours.

Since studies in this laboratory[10] have shown that significant therapeutic benefits in simian tuberculosis are associated with the continued maintenance of cycloserine blood concentrations of 25 γ per ml. or greater, it is interesting to note the relative time periods during which such concentrations were sustained in various animals used in this study. These periods, taken from figure 1, were as follows: mouse, 2 hours; guinea pig, 3¼ hours; rabbit, 10½ hours; monkey, 9¾ hours.

From the data presented in table 1, it is shown that cycloserine is eliminated rapidly in the urine of all animals used in this study, but that the rate of elimination was several times higher for the mouse than for the monkey, with the guinea pig and rabbit intermediate to these two. The results of this study also suggested

[15] The writers are indebted to Eli Lilly and Company for the cycloserine used in this study.

[16] Jones, L. R.: Personal communication to L. H. Schmidt.

[17] Jones, L. R.: Analyt. Chem., 1956, *28*, 39.

[18] Conzelman, G. M., Jr.: Antibiot. & Chemother., 1955, *5*, 444.

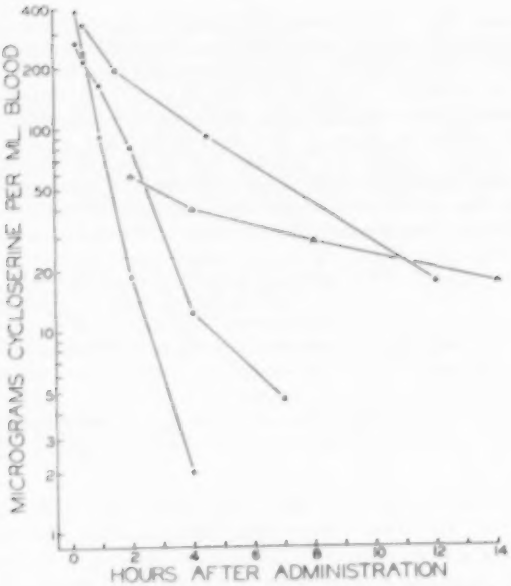

FIG. 1. Mean cycloserine concentrations in the blood of various laboratory animals after a single intramuscular injection.

×——× Mice—treated with 400 mg. per kg. of body weight (8 animals per point)

●——● Guinea pigs—treated with 200 mg. per kg. of body weight (2 animals per point)

○——○ Rabbits—treated with 200 mg. per kg. of body weight (serial observations on 3 animals)

△——△ Monkeys—treated with 50 mg. per kg. of body weight (serial observations on 6 animals)

TABLE 1

MEAN CUMULATIVE URINARY EXCRETION OF CYCLOSERINE IN VARIOUS LABORATORY ANIMALS AFTER INTRAMUSCULAR ADMINISTRATION

Animal	Number	Dose	Per Cent of Dose Recovered Hours After Administration				
			3	6	12	24	48
		mg. per kg.					
Mice	12	400	64.3	68.9	—	—	—
Guinea pigs	4	200	—	—	84.2	87.2	—
Rabbits	3	200	—	—	45.4	46.0	—
Monkeys	2	50	—	27.4	40.4	49.7	54.1

species differences in cycloserine metabolism which probably were not due wholly to differences in administered dose. Thus, the guinea pig metabolized only 13 per cent of a dose of 200 mg. per kg., whereas the rabbit metabolized 54 per cent of an equal dose.

Results of the chromatographic studies on urine specimens from treated rabbits indicated the presence of a substance which reacted with the nitroprusside reagent; this material was indistinguishable from the parent drug. Similar chromatograms on urine from treated mice and guinea pigs indicated the presence of a minor nitroprusside-reacting metabolite in addition to unchanged drug. This minor metabolite probably represented less than 10 per cent of the total chromogenic materials.

* * *

The data presented in this report have pointed to marked differences in the physiologic disposition of cycloserine in the various laboratory animals which have been used as test objects in experimental tuberculosis. These differences, as they relate to the maintenance of concentrations of cycloserine in the blood, appear to have a special bearing on the demonstrated effectiveness or ineffectiveness of this drug in the therapy of experimental and human tuberculosis.

At the present time, the only known definitive data relating the effectiveness of cycloserine to the concentration of this drug in the circulating blood are those acquired in studies in simian tuberculosis.[10] This work indicated that continued maintenance of blood concentrations in excess of 25 γ per ml. is a prerequisite to a significant therapeutic response in this infection. Such concentrations are maintained in the monkey receiving doses of 50 mg. per kg. twice daily. These are also maintained with the doses of cycloserine used in human tuberculosis, particularly in those studies in which the greatest therapeutic benefits have been reported. Despite administration of seemingly large individual doses, cycloserine concentrations in excess of 25 γ per ml. have probably not been maintained in those studies on mice, guinea pigs, and rabbits in which therapeutic responses have been questionable or limited. The data acquired in the present study (*see* figure 1) indicate that the single-dose regimen of 200 to 400 mg. per kg. used in treatment of mouse tuberculosis[3, 5] would provide minimal blood concentrations for less than 2 hours post treatment. The daily doses of 150 to 200 mg. per kg. employed in studies on guinea pigs[5, 12, 13] would provide effective concentrations for less than 3¼ hours, while the daily doses of 200 mg. per kg. used in work with rabbits[14] would give such concentrations for 10½ hours. It is noteworthy that limited therapeutic benefits in the rabbit were observed by Raleigh and associates[14] when doses of 200 mg. per kg. per day were administered.

The results of the excretion studies presented in this report indicate considerable differences in the rate of urinary elimination of cycloserine by the various animals and show that this rate was roughly parallel to the clearance of this drug from the blood. This finding suggests that the concentration of cycloserine in the blood is a good index of the over-all distribution of the drug in the body.

Data also shown in table 1 indicate that the animals studied in this investigation, like man[19] and monkey,[10] metabolized appreciable amounts of the administered dose of cycloserine to unknown substances. It will be noted, however, that

[19] Conzelman, G. M., Jr.: Federation Proc., 1956, *15*, 412.

drug metabolism varied from a low of 13 per cent in the guinea pig to a high of 54 per cent in the rabbit.

In summary: The physiologic disposition of cycloserine, administered intramuscularly to mice, guinea pigs, rabbits, and monkeys, has been studied. Half-life values for blood cycloserine ranged from a low of 23 minutes for the mouse to 7¾ hours for the monkey. The results of these clearance experiments suggest that, with the exception of the monkey, the blood concentrations obtained in these laboratory animals after even large daily doses of the drug may be too transitory to effect control of tuberculous infections.

Excretion data indicate that the rate of elimination of cycloserine by the kidney is roughly parallel to its clearance from the blood. These results also indicate that an appreciable amount of the drug is metabolized to unknown substances.

Chromatographic studies indicate that treatment urine samples from mice and guinea pigs contain a minor cycloserine metabolite which, like the parent drug, reacts with the nitroprusside reagent. Only unchanged cycloserine was detected in the urine from treated rabbits.

THE CHRIST HOSPITAL
INSTITUTE OF MEDICAL RESEARCH
CINCINNATI, OHIO

GAYLORD M. CONZELMAN, JR.
ROBERT KNAPP JONES

July 30, 1956

Correspondence

PSYCHIATRIC EVALUATION OF THE PERSONALITY OF THE TUBERCULOUS PATIENT

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

There has been an ever-growing interest in the personality pattern of the tuberculous patient on the part of both the psychiatrist and phthisiologist. It is to be hoped that the work which is being done in this particular field will eventually be of practical significance, especially in the handling of the patient who presents a difficult adjustment problem in the hospital. At present, it can be stated that these studies are of great interest, and further study in this direction has to be encouraged. However, as in any other relatively new field of investigation, there is a tendency to jump at conclusions at an early stage of the game.

The staff of our hospital had the privilege of attending a lecture given by one of the prominent West Coast psychiatrists, in which tuberculosis was labeled as a psychosomatic disease caused by a practically ubiquitous organism which affects only people of certain predisposition. The thoracic surgeons, by resecting the diseased portion of the lung, actually do not accomplish anything else but a cosmetic job on the chest film, because the patients will eventually develop the disease in some other parts of their lungs. These, and similar statements could be, per se, considered food for thought and a matter of discussion among specialists in tuberculosis and psychiatry. The unfortunate thing, however, is that these statements become known to lay people and present a very real practical problem.

A few days ago I was contacted by a business man who was considering employment of a patient soon to be discharged from the hospital. This man was on the verge of changing his mind concerning future employment of the patient, because he was told that the tuberculous are "mentally disturbed" people. It is believed that the stigma of tuberculosis is great enough to cope with without the additional and still worse stigma of mental disease.

Due to the widespread popular glamour which psychiatry enjoys nowadays, this type of information gets to the lay public very easily by a process of diffusion through nurses, social workers, occupational therapists, medical secretaries, et cetera. It is easy to see the potential harm which this may do to the rehabilitation of the tuberculous patient. It is to be hoped that the National Tuberculosis Association in cooperation with the American Psychiatric Association can work out something to prevent premature publicity of these studies.

VETERANS ADMINISTRATION REGIONAL OFFICE
ROANOKE, VIRGINIA

L. S. ARANY

June 20, 1956.

METHANOL EXTRACTS OF TUBERCLE BACILLI

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

Recently[1] R. J. Dubos, D. W. Weiss, and R. W. Schaedler published an article on "The Enhancing Effect of Adjuvants on the Antituberculous Immunity Elicited in Mice by Methanol Extracts of Tubercle Bacilli."

[1] DUBOS, R. J., *et al.*: Am. Rev. Tuberc., 1956, *73*, 781.

I would be grateful to you if the following comment to this article could be published in the REVIEW.

Immunizing properties of methanol extracts from acetone-defatted tubercle bacilli were demonstrated by A. Boquet and myself many years ago.[2] I am glad to note that R. J. Dubos and his collaborators have been able to confirm our findings and have adopted a method different from ours only in the use of phenol instead of heating for the sterilization of tubercle bacilli; they also add adjuvants for the reinforcement of the immunizing qualities of the extract.

We have shown[3] that, even without the addition of such adjuvants, guinea pigs and rabbits, injected with strong doses of methanol extracts of acetone-defatted tubercle bacilli, acquired an antituberculous resistance comparable with the one obtained with BCG but without allergy.

Recently, J. Bretey and I[4] have found a delay in the dispersion of superinfected virulent bacilli in the viscera of guinea pigs and rabbits which were treated two weeks previously with our methanol extracts comparable to the delay that is observed after BCG vaccination, the only difference being the shorter duration of antituberculous resistance in the case of methyl antigen than after BCG.

For this reason, we do not recommend methyl antigen for the prevention of tuberculosis in man. We found, however, that animals, when vaccinated with BCG and then treated with repeated injections of methyl antigen, show more pronounced antituberculous resistance than animals vaccinated with BCG alone.[5] In consequence, our methanol extracts appear to reinforce the immunizing activity of BCG.

L. NÈGRE

INSTITUT PASTEUR
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PARIS XV^{e}, FRANCE

August 6, 1956

INCIDENCE OF TUBERCULOUS INFECTION IN INFANCY

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

In the July issue of the REVIEW, Dr. Edwin L. Kendig, Jr., reported his observations concerning the incidence of tuberculous infection and subsequent tuberculous disease in Negro children in Richmond, Virginia. In 791 randomly selected children from a well-baby clinic, Dr. Kendig found that 13.5 per cent became infected with tuberculosis before they reached the age of three years. Further, among the 121 children who were infected before the age of five years only two developed "complications of the disease": one a tuberculous pneumonia, the other tuberculosis of a superficial cervical lymph node.

These observations are in sharp disagreement with those made by other authors in similar studies, and which have formed the basis for two widely held concepts in the field of tuberculosis: that the present incidence of tuberculous infection in children in the United States is low, even in Negroes, and that such an infection, when it occurs in

[2] BOQUET, A., AND NÈGRE, L.: Compt. rend. Soc. de biol., 1920, *83*, 960; 1922, *87*, 1162; Compt. rend. Acad. d. sc., 1924, *178*, 891; Ann. Inst. Pasteur, 1923, *37*, 787; 1930, *95*, 415.

[3] NÈGRE, L.: Ann. Inst. Pasteur, 1950, *134*, 445.

[4] NÈGRE, L., AND BRETEY, J.: Bull. Acad. nat. méd., Paris, 1955, *139*, 288.

[5] NÈGRE, L., AND BRETEY, J.: Ann. Inst. Pasteur, 1952, *93*, 429.

children under three years of age, is a serious event, not infrequently leading to clinical tuberculous disease.

Testing in Kansas City in 1945, Furcolow[1] found positive tuberculin reactions[2] in 5.3 per cent of Negro children in the age group 4–6 years. In the Eastern Health District of Baltimore, 6.6 per cent of Negro children in the same age group, living mostly in overcrowded dwellings of poor hygienic standard, were found to be reactors to tuberculin[3] in the years 1951–1954, a period during which the incidence of reported tuberculous disease was considerably higher in Baltimore than in Richmond.[4] Compared with findings like these, the infection rate as reported by Dr. Kendig in even younger children seems surprisingly high. It is an infection rate which would be expected only in children living in close contact with cases of tuberculosis. In such children, however, one would also expect to find a high incidence of tuberculous disease. To use eastern Baltimore again as an example, Brailey[5] found that 42 per cent of tuberculin-positive Negro children under three years of age had parenchymal pulmonary lesions detected by their initial roentgenograms.

The question which presents itself in connection with Dr. Kendig's report, his discussion of which would be of great interest, is the following: Is it possible that a majority of the conversions observed by Dr. Kendig were acquisitions of nonspecific tuberculin sensitivity, without relation to tuberculous infection?

As demonstrated by Furcolow and his associates,[6] almost all persons react to tuberculin provided sufficiently large doses are given. It has been shown[7] that tuberculin sensitivity brought out only by doses of 100 TU or more is largely nonspecific, being unrelated to history of exposure but highly dependent on geographical location. Recent studies[8] indicate that nonspecific sensitivity is manifested even with doses as low as 5 TU of PPD and that in southeastern states perhaps one-third of the positive reactions observed to this dose might be unrelated to tuberculous infection.

Dr. Kendig employed 1.0 mg. of Old Tuberculin in about half of his cases and 0.1 mg. in nearly all. Assuming that the tuberculin employed corresponded in potency to the International Standard, a dose of 1.0 mg. (100 TU) must be considered entirely unsuited for the separation of infected individuals from those not infected, in any southeastern state. Even 0.1 mg. (10 TU) would presumably elicit a large number of nonspecific reactions so that the minimal size of a positive reaction would have to be set large in order to preserve the specificity of the test.

The evaluation of the high incidence of infection and low incidence of disease reported by Dr. Kendig will be possible only with information on his criteria for "infected with tuberculosis" and on the actual size of the reactions observed.

615 N. Wolfe Street
Baltimore 5, Md.

Edward F. Krohn

July 19, 1956

[1] Furcolow, M. L., *et al.*: Pub. Health Rep., 1946, *61*, 1132.

[2] 5 mm. or more of induration to 0.0001 mg. of PPD-S (5 TU).

[3] 5 mm. or more of induration to 0.01 ml. of Old Tuberculin (approximately 1.5 TU).

[4] Krohn, E. F.: An analysis and interpretation of the results of a study of tuberculin reactions in school children in Baltimore, The Johns Hopkins University, School of Hygiene and Public Health, Thesis, 1952 (unpublished).

[5] Brailey, M.: Am. J. Pub. Health, 1943, *33*, 343.

[6] Furcolow, M. L., *et al.*: Pub. Health Rep., 1941, *56*, 1082.

[7] WHO Tuberculosis Research Office: Bull. WHO, 1955, *22*, 63.

[8] Palmer, C. E., *et al.*: Pub. Health Rep., 1956, *71*, 633.

TEST OF PAS INGESTION

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

Owing to the fact that some patients tolerate PAS poorly, they have been known to refrain from taking the drug without informing their physician. The following test is useful as a rapid screening method to check whether the drug is being taken.

This test, which is adapted from the method of Short (SHORT, E. I.: Biochem. J., 1951, *48*, 301), depends upon the reaction of *p*-dimethylaminobenzaldehyde with the free arylamino group of PAS to give a yellow compound.

A urine specimen is procured from the patient two to four hours after the dose of PAS has presumably been taken. In many cases a positive result is obtained even after a twelve-hour interval.

Test: To 8.8 ml. of 4 per cent trichloroacetic acid add 0.2 ml. of urine.
Add 1 ml. of color reagent. Mix well.
The presence of PAS is indicated by a bright yellow color.

Reagents: Trichloroacetic acid—4 per cent.
Color reagent: Dissolve 2 gm. of *p*-dimethylaminobenzaldehyde in 100 ml. of distilled water. To this add 100 ml. of 4M sodium acetate.

Using the above quantities, the test may be used quantitatively by centrifuging to remove any protein precipitated by the trichloroacetic acid and reading in a spectrophotometer at a wavelength of 450 mu. A reagent blank is necessary and a calibration curve is made, using suitable dilutions of PAS. Quantitatively the method will detect PAS in amounts ranging down to 1 mg. per 100 ml. of urine.

We have found no interference from salicylates such as aspirin, although a positive result will be obtained in the presence of sulfonamides.

D. K. PARROTT

TORONTO HOSPITAL FOR
TUBERCULOSIS
TORONTO, ONTARIO

July 30, 1956

PROPER DESIGNATION OF AMMONIUM SULFATE PPD

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

Purified protein derivative (PPD), prepared by the trichloroacetic acid precipitation of cultures of tubercle bacilli grown on synthetic medium, has not been commercially available in the United States for many years. Instead, purified protein derivative prepared by the ammonium sulfate precipitation of cultures of tubercle bacilli grown on synthetic medium has been commercially available and has continued to be labeled by the manufacturers as PPD. Since these two preparations differ in their chemical, physical, and biologic properties, it would seem necessary to differentiate them by a specific designation. This is important in view of the fact that the trichloroacetic acid PPD continues to be prepared by the State Serum Institute, Copenhagen, and by the Ministry of Agriculture and Fisheries, Weybridge, Surrey, England. Since the trichloroacetic acid PPD is used abroad, it is not clear from the publications which purified protein derivative is used. I, therefore, suggest that the ammonium sulfate purified protein derivative be given a specific

designation to differentiate it from the original trichloroacetic acid purified protein derivative (PPD).

HENRY PHIPPS INSTITUTE
UNIVERSITY OF PENNSYLVANIA
PHILADELPHIA 47, PENNSYLVANIA

Sincerely yours,
JOSEPH D. ARONSON

August 14, 1956

THE FINDING OF LUPUS ERYTHEMATOSUS CELLS IN SARCOIDOSIS

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

In view of the altered plasma protein relationship with frequent reversal of the albumin-globulin ratio noted in cases of sarcoidosis, we thought it would be of great interest to search for lupus erythematosus cells in the peripheral blood.

Accordingly, a random sampling was made of 27 cases of sarcoidosis followed at the Bedford Chest Clinic of the New York City Department of Health. The patients had a history which suggested comparatively recent onset. The diagnosis of sarcoidosis had been proved by clinical findings, roentgenograms, positive Kveim tests, and node biopsies. The total proteins and albumin-globulin ratio were performed in only a few cases. The search for lupus erythematosus cells was made at the diagnostic clinic under the directorship of Dr. Anthony Bianco.

The lupus erythematosus phenomenon was not seen in the peripheral blood in any of the 27 cases of sarcoidosis. Thus, the altered protein metabolism in sarcoidosis does not give rise to the findings of lupus erythematosus cells and so there can be no confusion with a diagnosis of lupus erythematosus.

BEDFORD CHEST CLINIC, BUREAU OF TUBERCULOSIS
NEW YORK CITY DEPARTMENT OF HEALTH
NEW YORK, NEW YORK

EMANUEL SCHWARTZ

August 15, 1956

Books

A History of the Therapy of Tuberculosis and the Case of Frédéric Chopin. By Esmond R. Long. Pp. 71, University of Kansas Press, Lawrence, Kansas, 1956. Price, $2.00.

A case history viewed in retrospect serves as the prelude to this concise résumé of tuberculosis treatment from the days of Hippocrates to isoniazid. The material was first presented as one of the Logan Clendening Lectures on the History and Philosophy of Medicine (sixth series) at the University of Kansas School of Medicine.

The case history is that of Frédéric Chopin who died of tuberculosis in 1849 at the age of thirty-nine and whose death, as the author points out in the preface, exemplifies the helplessness of earlier days in contrast to recent progress in treating tuberculosis. In this particular case, however, the suffering of the victim was not completely in vain since, while violating all the rules of a later, better informed era, he gave the world what the author calls "some of its most treasured musical compositions."

The second part of the book consists of a compact history of the prevailing medical attitude toward tuberculosis from earliest written history to the present. The volume affords a brief yet comprehensive historical record of tuberculosis therapy, with sketches of men whose contributions to the treatment of this ancient disease led to the present era of chemotherapy in the hands of skilled practitioners—and to hope on the part of the patient. The book is written in the easy, clear style characteristic of the author.

Reading of many of the "cures" of other days, one marvels not so much that tuberculosis took so many lives but that any patient survived such treatment as copious blood-letting, strenuous exercise, and a variety of practices which seem more akin to superstition than to science. Progress has been made in treatment, but, as for the disease itself, the author states, "tuberculosis had not changed in fundamental character since ancient times."

Agnes Fahy
New York, New York

La Tuberculose Primarie Chez l'Enfant. By Raymonde Grumbach. Pp. 92, with illustrations, Expansion Scientifique, Paris, 1955.

The study by Mlle. Grumbach is a report on the pathology of primary tuberculosis in children who died during treatment for tuberculous meningitis. The data are derived from examination of 105 patients. In the preface, Professor Debré points out the opportunity offered in this study to reconstruct the course of primary tuberculous lesions during antibiotic treatment from the onset to final healing. The book is divided into five interesting chapters. The figures are clear and instructive.

Since the treatment of primary tuberculosis is now topical in pediatrics, Dr. Grumbach's book is recommended to members of this clinical specialty as well as to all students of tuberculosis.

Ole Wasz-Höckert
Helsinki, Finland

BOOKS RECEIVED

Cardiac Diagnosis. A Physiologic Approach. By Robert F. Rushmer. Pp. 447, W. B. Saunders Company, Philadelphia, 1955. Price, $11.50.

STATISTICS OF THERAPEUTIC TRIALS. By G. Herdan. Pp. 367, D. Van Nostrand Company, Inc., Princeton, N. J., 1955. Price, $10.50.

CARDIOVASCULAR SURGERY. Proceedings of International Symposium on Cardiovascular Surgery held at Henry Ford Hospital, Detroit, Mich. Pp. 543, W. B. Saunders Company, Philadelphia, 1955. Price, $12.75.

TUBERKULOSE-JAHRBUCH. By Rolf Griesbach. Pp. 283, Springer-Verlag, Berlin, Germany, 1956.

REHABILITACION DEL TUBERCULOSO PULMONAR. By Ermilo Esquivel Medina. Pp. 98, Comité Nacional de Lucha Contra la Tuberculosis, Mexico, 1956.

CURRENT THERAPY 1956. Edited by Howard F. Conn. Pp. 632, W. B. Saunders Company, Philadelphia, 1956. Price, $11.00.

American Trudeau Society

Medical Section of the National Tuberculosis Association

The Present Status of Skeletal Tuberculosis

A Statement of the Committee on Therapy

The treatment of skeletal tuberculosis depends upon an adequate understanding of the pathogenesis of the disease.

Tuberculosis of the skeletal system results from the hematogenous spread of infection from tuberculous disease elsewhere. There is evidence to suggest that some lesions of the spine and thoracic cage may develop from direct extension from contiguous caseous lymph nodes. The frequent occurrence of antecedent pleurisy with subsequent paravertebral lymph node involvement is cited in support of this theory. In any event, bone and joint lesions are secondary manifestations developing in a variable period of time—from weeks, months, or even years after the primary. With few exceptions, the primary infection is by inhalation and is pulmonary.

Bone and joint tuberculosis is most likely to occur in infancy and early childhood. In adults, the incidence is higher in Negroes and in males generally. In the past, approximately 4 per cent of tuberculous patients had skeletal involvement. Of these, usually between 45 and 50 per cent had associated clinical pulmonary tuberculosis. Currently the incidence of the disease is probably falling. This may be due to a later occurrence of initial tuberculous infection and a somewhat reduced tendency to severe hematogenous disseminations. In the past decade, probably because of earlier diagnosis, associated pulmonary disease has been less advanced.

The end results of therapy depend considerably on an early diagnosis. One should not overlook the possibility that more than one lesion exists concurrently. The diagnosis of bone and joint tuberculosis may be suggested by clinical means, but it is confirmed by several routine tests. The tuberculin test (Mantoux) should be performed initially since, if negative, it rules out tuberculosis.

Local signs of inflammation of a joint usually are associated with an increase in fluid. Aspiration of this fluid, if reasonably accessible for diagnostic study, should be done promptly. The results of microscopy and culture for tubercle bacilli will vary. Usually, purulent secretions are most likely to be positive. Results as high as 87 per cent positive have been reported in presence of frank pus. A single negative smear or culture does not exclude the diagnosis. Biopsy of the synovial membrane should be considered if the diagnosis remains in doubt. This affords opportunity for both histologic and bacteriologic study. Unfortunately, examination of the material is not always positive when the condition is tuberculous, and the biopsy may have to be repeated before the diagnosis is established.

Anterior-posterior and lateral roentgenograms of the involved joint contribute tremendously to the diagnosis, plan of treatment, and estimate of prognosis. If roentgenograms are obtained very early, there may be no evidence of disease except soft tissue swelling. The first discernible lesion in most bones is associated with demineralization and later with small areas of erosion. These destructive lesions are usually located in the metaphysis immediately subchondrally. Characteristically, the destructive lesions are associated with little evidence of periosteal reaction or new bone production unless secondary infection has occurred through a sinus tract. Small destructive cortical lesions

may be missed by conventional roentgenograms and may only be demonstrated by serial laminagrams. Progressive erosion of cortical bone is accompanied by cartilaginous destruction. This damage usually proceeds in the nonarticulating areas first, finally progressing to complete loss of joint space and total disorganization. In advanced neglected lesions, abscesses may develop which may burrow into surrounding tissue. Various amounts of calcification may be evident.

Roentgenographic changes in spinal tuberculosis may initially be shown as slight loss of intervertebral space. Later, demonstrable destructive lesions of the adjacent vertebral body may be seen. Usually, the greatest amount of destruction is anterior.

Multiple involvement of vertebral bodies may occur and, in advanced lesions, there may be varying degrees of angulation—as the spine settles forward with resulting gibbus formation. Involvement of transverse or spinous processes is rare. Paravertebral abscess is a late finding, being observed in about 75 per cent of the patients. It may extend a considerable distance along the psoas sheath. Spinal tuberculosis does not lend itself readily to direct examination, and the diagnosis, therefore, depends substantially on roentgenographic examination. Early loss of interspace with anterior destruction and collapse of vertebral body contrasts markedly with metastatic malignancy in which extensive vertebral body damage with late collapse is the rule. Nucleus pulposus injury is usually posterior at the level of the interspace, but may require the introduction of contrast media into the cerebrospinal fluid to establish the diagnosis.

The treatment of skeletal tuberculosis is both general and local. Bed rest, general supportive measures, and chemotherapy should be instituted as soon as possible. In approximately half of the patients, pulmonary disease is present and should receive prior consideration until some measure of control has been established. Concurrent orthopedic measures to treat the bone and joint lesions should be started immediately, but it is wise to defer major surgery until the clinical course of the pulmonary disease is satisfactory. When no other manifest disease exists, the prognosis is usually better, but the principles of general supportive treatment and chemotherapy still apply.

Chemotherapy is the most important therapeutic measure affecting the course of bone and joint tuberculosis and should be used in all cases for a prolonged period of time. It has reduced mortality by two-thirds and accelerated the rate of recovery. Some patients with slight damage, particularly children, have recovered with apparently normal joints without needing surgery. Many who would have been "hopeless" have qualified for orthopedic surgery. Surgery has been performed earlier and with smoother postoperative course. Draining sinuses of long duration have closed under its use. Amputations are rarely justified.

Isoniazid[1] is the drug of first choice because of its diffusibility and greater antituberculous activity. It is usually given concurrently with streptomycin or PAS uninterruptedly for a minimum of eighteen months or longer.

The drug regimens and duration of treatment that have become accepted as satisfactory for pulmonary and for renal disease should be employed in the treatment of bone and joint tuberculosis. The following drug regimens are considered to be satisfactory:

1. *Streptomycin-PAS:* 1.0 gm. of streptomycin intramuscularly two or three times weekly and 12 gm. of PAS (or 15 gm. of sodium PAS) daily by mouth in three or four divided doses
2. *Isoniazid alone:* 5.0 mg. or more of isoniazid per kg. of body weight per day in two or three divided doses

[1] Iproniazid has been used in the treatment of skeletal tuberculosis, but has not been generally accepted because of its toxicity.

3. *Isoniazid-streptomycin (both daily)*: 5.0 mg. or more of isoniazid per kg. and 1.0 gm. of streptomycin intramuscularly
4. *Isoniazid daily—streptomycin twice weekly:* Same dosage as in regimen *3*, but streptomycin given twice weekly instead of daily
5. *Isoniazid-PAS:* 5.0 mg. or more of isoniazid per kg. per day and 12.0 gm. of PAS daily
6. *Isoniazid-streptomycin-PAS:* each in dosage as above, streptomycin given daily or twice weekly.

Regimens *1* and *2*, streptomycin-PAS and isoniazid alone, appear to be somewhat less effective than the other regimens, as judged by organized cooperative trials in pulmonary tuberculosis. Percentage advantages in favor of regimens *3* to *6*, in terms of such clinical criteria as roentgenographic improvement and sputum culture "conversion," are consistently found in various reports of large-scale comparative regimen trials. These advantages, however, are not large and other factors may be considered in selecting a regimen for a given patient with pulmonary and/or extrapulmonary tuberculosis. Regardless of the regimen selected, therapy should be continuous and for a minimal period of eighteen months or longer.

The success of current chemotherapy should not imply the abandonment of many orthopedic measures, both surgical and nonsurgical, in the treatment of skeletal tuberculosis. Even in the most favorable cases, there may be distention of the joint with fluid, and usually this should be aspirated both for diagnostic and therapeutic reasons. Needle tract sinuses seldom occur with chemotherapy. Abscesses should be aspirated whenever they are reasonably accessible. Deep-seated psoas or paravertebral abscesses are treated conservatively unless they increase in size or are thought to be the cause of symptoms or signs, such as paraplegia. Debridement with tight closure of the incision is performed by some surgeons for some of these deep-seated abscesses.

Prior to major surgery, immobilization by frame, cast, brace, or sling of the affected part is usually carried on until clinical improvement is evident. An attempt may be made to overcome by traction some of the flexion or adduction deformities which may have developed with lower extremity lesions. Weight-bearing should be avoided to prevent trauma, even though in children complete restraint with cast may not be required. Under these latter circumstances, limited nonweight-bearing exercise probably prevents undue atrophy of the extremity and avoids premature epiphyseal closure which may occur with disuse. In the spine, if paraplegia exists, an immediate attempt to relieve it should be made by placing the patient on a frame. If recovery does not occur promptly, i.e., within two to four weeks, exploration to evacuate paravertebral abscesses is justified.

The decision regarding surgery involves chiefly the question of whether an arthrodesis should be done. It will depend on several factors, and in certain joints, such as the knee, may properly be deferred four to six months to await improvement under chemotherapy when there is doubt. Serious pulmonary or other disease may delay the decision even longer.

The choice of surgical procedures will differ considerably among surgeons. Both intra- and extra-articular procedures have been advocated in the treatment of the same lesion. Many variations of a few basic procedures exist and a number provide acceptable results, with solid mechanical fusion and the involved parts fixed in a useful attitude. A fairly liberal use of bone grafting seems evident currently, and the results justify it. This practice has been aided, no doubt, by chemotherapy. In recent years, the use of refrigerated or Co^{60} irradiated bone-bank bone has been more frequently employed for

the grafting of large areas in adults, or in children in whom limited autogenous bone is available.

Conclusion: Treatment of skeletal tuberculosis is a joint responsibility of the internist and orthopedic surgeon.

Early in the course of treatment, a joint plan of therapy should be formulated which would include all aspects of general as well as local treatment. This plan should be rechecked periodically to obtain the best result.

Chemotherapy has vastly improved the prognosis and reduced morbidity and mortality. It should be given without interruption up to eighteen months to two years. Its effect has been to make surgery much safer and with fewer postoperative difficulties. Some persons, particularly children, have escaped the need for fusion procedures. Amputations are greatly reduced.

Early diagnosis offers the hope that additional lesions may be spared surgical intervention. A high degree of suspicion regarding possible tuberculosis must be maintained if these lesions are to be brought under proper treatment early.

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Obituaries

EDGAR M. MEDLAR

1887–1956

Edgar M. Medlar, great research pathologist, long-time laboratory chief, former teacher, American Trudeau Award winner, and quiet good friend of hundreds of his colleagues, died at Tupper Lake, New York, on June 30, 1956, at the age of sixty-nine years.

Dr. Medlar was a native of Nebraska, and received collegiate degrees from Doane College and the University of Nebraska. He earned his medical doctorate from Harvard University in 1913, and was pathologist for four years at the Boston City Hospital. After service in the Medical Corps during World War I he was a faculty member in pathology at the University of Wisconsin and at Columbia University. He then became director of the Hegeman Memorial Research Laboratory at Mt. McGregor Sanatorium near Glens Falls, New York, operated by the Metropolitan Life Insurance Company, and he remained there for twenty-five years. He served on the staff of the Sunmount Veterans Administration Hospital at Tupper Lake from 1948 to 1951; became principal pathologist for the New York State Tuberculosis Service at the Hermann Biggs Memorial Hospital in Ithaca from 1951 until its recent closure; and was about to rejoin the Sunmount Veterans Administration Hospital staff when he had a first heart attack a few weeks before his death.

It would be hard to decide which of his many accomplishments Dr. Medlar would favor most, since he considered each to be a step or building stone. He felt that facts were basic; that each piece of work must stand by itself once he had done it and given his best interpretation; and he expected criticism and disbelief, quietly waiting for the time when, as all good pathologists firmly believe, their word will be final and correct. That time almost always arrived, although it often took years.

Dr. Medlar's personal load was a heavy and recurrent one, but it rarely slowed his constant pace. He did the many thousands of serial sections which demonstrated the bilateral nature of tuberculous nephritis with a nagging formalin dermatitis of his hands. He survived the removal of a huge hypernephroma in the early 1930's, and went on with his interpretation of the pathology of tuberculosis, and its reflection in the leukocyte count, while suffering from a severe neurovascular disturbance after abdominal radiation. He described the effect of posture in animals on the location of tuberculosis while in the early stages of a rheumatoid arthritis; worked with the clinicians on the effect of chemotherapy on healing of lung lesions while he was still ill; described the nature of the lesions found in the apical fibrosis of accident victims while in a wheel chair; and became ambulatory on arm-crutches while explaining his work on tuberculosis and bronchiectasis, and while writing a supplement for the AMERICAN REVIEW OF TUBERCULOSIS AND PULMONARY DISEASES. A summary of his writings is fundamental reading for a modern knowledge of the disease. He received the Trudeau Medal in 1954, and was named an honorary member of the Canadian Tuberculosis Association this spring.

Dr. Medlar is survived by his widow; by a son, Dr. Robert E. Medlar of Jackson, Michigan; and by a daughter, Mrs. James Brewton of Long Island.

W. H. O., Jr.

FRANCIS B. TRUDEAU, SR.

1887–1956

Francis B. Trudeau, Sr., died of a heart attack at the age of sixty-nine years in his boat on Upper St. Regis Lake on July 19, 1956. The setting was in the family tradition, in which an outdoor existence in the Adirondacks has been the mode for generations.

Dr. Trudeau was born in Saranac Lake, practiced there since his service as a captain in the Medical Corps in World War I, and has been well known for his interest in medical and public affairs and for his sympathetic help to patients. He was a son of Dr. Edward Livingston Trudeau, whose name the American Trudeau Society bears, and who was the founder of Trudeau Sanatorium seventy-two years ago.

Francis Trudeau graduated from Yale in 1909, received his M.D. degree from Johns Hopkins University in 1913, and married Miss Helen Garretson the following year. They had two sons, Dr. Francis B. Trudeau, Jr., who has followed his father's practice and activities, and Edward L. Trudeau of Albany.

Dr. Trudeau became a member of the Board of Trustees of Trudeau Sanatorium in 1919, was Chairman of its Medical Board for eight years after 1937, and served as President of the Board of Trustees of the Sanatorium and Foundation from 1945 to 1952. He also served as a recorder for the American Clinical and Climatological Association from 1940 to 1946; was a director of the National Tuberculosis Association; was a member of the New York State Board of Welfare; and was President of a Saranac Lake tuberculosis control group, two medical societies, the Rotary Club, a fish and game club; and was an officer of two Episcopal churches in the Saranac area.

W. H. O., Jr.

CHARLES J. KAUFMAN

1897–1956

Dr. Charles J. Kaufman, chief of the tuberculosis service at the Veterans Administration Hospital, Castle Point, died in the New York Presbyterian Hospital on June 15, 1956, at the age of fifty-nine years. He was a native of New York; graduated from City College and the Cornell University Medical School; served in the pulmonary division of the New York Hospital clinic; and was a former faculty member of the medical schools at Cornell and the University of Colorado. He conducted a tuberculosis survey of New Jersey state hospitals for the Department of Public Health and Welfare in 1930–1931. He engaged in private practice in New York for six years and then was Medical Director of the National Jewish Hospital at Denver for ten years. He then went to Castle Point where he was active until he entered the hospital for surgery two months before his death.

Dr. Kaufman was a Fellow of the New York Academy of Medicine and a member of the American Trudeau Society, the American College of Chest Physicians, and the American Medical Society. Surviving are his widow, Rosalie, a son, Michael, and a daughter, Margaret.

W. H. O., Jr.

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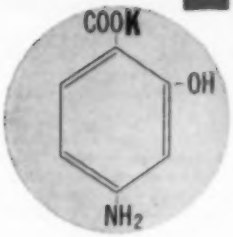

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1. *Molthan, L., Cohen, R. V., and Zarafonetis, C. J. D.: Clinical use of potassium para-aminosalicylate (KPAS). Am. Rev. of Tuberc. and Pulmon. Dis. 71:220 (Feb.) 1955.*

2. *Cohen, R. V., Molthan, L., and Zarafonetis, C. J. D.: Clinical Studies of Various Forms of PAS (with special reference to plasma concentrations). Read before the American Trudeau Society, Annual Meeting, Milwaukee, Wisconsin, May 23-27, 1955.*

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